IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants: Campbell Rogers, Elazer R. Edelman, and Daniel I. Simon

Serial No.:	08/823,999	Art Unit:	1644
Filed:	March 25, 1997	Examiner:	Phillip Gambel

For: *MODULATION OF VASCULAR HEALING BY INHIBITION OF LEUKOCYTE ADHESION AND FUNCTION*

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REPLY TO ORDER RETURNING UNDOCKETED APPEAL TO EXAMINER

Sir:

Responsive to the Order Returning Undocketed Appeal To Examiner mailed November 17, 2005, Appellants submit copies of references listed below, which were relied on by the examiner on pages 4-6 of the Examiner's Answer mailed December 9, 2004 in response to the Substitute Appeal Brief filed August 10, 2004, for the above-referenced application.

ALTIERI, et al., *J. Biol. Chem,.* 268(3):1847-1853 (1993).

ANDERSON, *Disease-a-month,* 39(9):617-670 (1993)

BENDECK, et al., *J. Vasc. Res.,* 38:590-599 (2001).

DANGAS, et al., *Am Heart J.,* 132:428-436 (1996).

DIAMOND, et al., *J. Cell Biol.*, 130:1473-1482 (1995).

FATTORI, et al., *Lancet*, 361:247-249 (2003).

FAXON, et al., *J. AM. Coll. Cardiol.*, 40:1199-1204 (2002).

HEMKER, et al., *Emerging Drugs*, 4:175-195 (1999).

IKEDA, et al., *Am. Heart J.*, 128:1091-1098 (1994).

INOUE, et al., *JACC,* 28:1127-1133 (1996).

KLING, et al., *Circulation Research*, 77:1121-1128 (1995).

KLING, et al., *Arteriosclerosis and Thrombsis*, 12:997-1007 (1992).

KUNTZ, *Science*, 257:1078-1082 (1992).

MAZZONE, et al., *Circulation*, 88:358-363 (1993).

PIMANDA, et al., *Curr. Drug Targets Cardiovasc, Haematol. Discord,* 3(2):101-123 (2003).

ROGERS, et al., *Circulation* 88:1215-1221 (1993).

SCHWARZ, et al., *Thrombosis Research*, 107:121-128 (2002).

WELT, et al., *Arterioscler Throm Vasc Biol*, 22:1769-1776 (2002).

WU, et al., *Thrombosis Research*, 101:127-138 (2001).

SIMON, et al., *Circulation*, 100(Suppl 1)(18): #1742, 11/2/99 (Exhibit)

Taber's Cyclopedic Medical Dictionary, 18th Ed., pages 130, 1666 and 1828 (1997).

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REPLY TO ORDER RETURNING UNDOCKETED APPEAL TO EXAMINER

The enclosed references are submitted in response to matters requiring attention prior to docketing, identified in the above-mentioned Order Returning Undocketed Appeal to Examiner.

Respectfully submitted,

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Date: November 28, 2005

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The Structural Motif Glycine 190-Valine 202 of the Fibrinogen γ Chain Interacts with CD11b/CD18 Integrin ($\alpha_M\beta_2$, Mac-1) and Promotes Leukocyte Adhesion*

(Received for publication, July 21, 1992)

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The leukocyte-restricted integrin CD11b/CD18 ($\alpha_M\beta_2$, Mac-1) is a receptor for fibrinogen on stimulated monocytes and neutrophils. At variance with platelet $\alpha_{IIb}\beta_3$ or endothelial cell $\alpha_v\beta_3$ integrins, CD11b/CD18 interacts with a ~30-kDa plasmic fragment D (D_{30}) of fibrinogen that lacks the Arg-Gly-Asp sequences in the Aα chain and the carboxyl terminus of the γ chain. Using epitope-mapped antibodies and synthetic peptidyl mimicry, we have now identified a unique linear sequence in fibrinogen that mediates ligand binding to CD11b/CD18. Anti-fibrinogen antibodies directed to the γ chain region 95–264 inhibited ^{125}I-fibrinogen or ^{125}I-D_{30} binding to chemoattractant-stimulated neutrophils or monocytic THP-1 cells in a dose-dependent fashion. Partially overlapping synthetic peptides reproducing this γ chain region were tested for their ability to inhibit fibrinogen binding to leukocytes. A synthetic peptide designated P1, duplicating γ chain Gly^{190}-Val^{202}, inhibited ^{125}I-fibrinogen binding to stimulated neutrophils or THP-1 cells and blocked adhesion of these cells to immobilized fibrinogen in a dose-dependent fashion. Increasing concentrations of P1 inhibited ^{125}I-fibrinogen binding to isolated CD11b/CD18 in a cell-free system. Consistent with genuine peptidyl mimicry, ^{125}I-P1 bound saturably to THP-1 cells in a reaction inhibited by molar excess of unlabeled peptide, fibrinogen, or D_{30}. Finally, immobilized P1 effectively supported adhesion of THP-1 cells in a CD11b/CD18-dependent manner. These data suggest that the fibrinogen γ chain region Gly^{190}-Val^{202} functions as a minimal recognition sequence for the leukocyte integrin CD11b/CD18. Given the participation of fibrinogen:leukocyte interaction in inflammation and atherogenesis, antagonists based on this unique structural motif would effectively interfere with aberrant leukocyte adhesion mechanisms without affecting Arg-Gly-Asp-directed vascular integrins.

Leukocyte β_2 integrins mediate a variety of cell:cell and cell:substratum adhesive interactions during immune-inflammatory responses (reviewed in Refs. 1 and 2). Ubiquitously expressed on all leukocytes of lymphoid and myelo-monocytic lineage, these differentiation molecules are structurally composed by a common β subunit CD18 that associates with three unique α subunits, CD11a (α_L), CD11b (α_M), and CD11c (α_X) to form the heterodimers LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18) (3). Through a regulated mechanism of ligand binding that involves both soluble as well as cell-associated ligands (1, 2), CD11/CD18 integrins contribute to leukocyte adherence to resting or inflamed endothelium, T and B lymphocyte effector interactions, phagocytosis, and propagation of the coagulation and complement cascades (reviewed in Refs. 4 and 5). Similar to other integrins (1, 2), optimal ligand binding to CD11/CD18 molecules requires a transient high avidity state of the receptor, which is produced by various inflammatory agonists (6, 7) or by favorable engagement of the divalent ion binding site(s) (8, 9).

On stimulated monocytes and polymorphonuclear leukocytes (PMN),[1] CD11b/CD18 integrin functions as a high affinity receptor for fibrinogen (10–13). This reaction appears to be mediated by a novel structural interacting motif in the ligand. As contrasted to the recognition of other vascular cell integrins on platelets ($\alpha_{IIb}\beta_3$) (14) or endothelial cells ($\alpha_v\beta_3$) (15), CD11b/CD18 interacts with an ~30-kDa plasmic fragment D of fibrinogen (D_{30}) that lacks the Arg-Gly-Asp sequences in the Aα chains and the COOH terminus aspect of the γ chain (16). Using epitope-mapped antibodies and synthetic peptidyl mimicry, we have now identified a candidate region in the fibrinogen γ chain that interacts directly with CD11b/CD18 and modulates its receptor:ligand binding properties.

MATERIALS AND METHODS

Cells and Cell Culture—The monocytic cell line THP-1 (American Type Culture Collection, Rockville MD) (17) was maintained in continuous culture in RPMI 1640 medium (M. A. Whittaker Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (Whittaker), 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA), 10 mM Hepes, and 10^{-5} M 2-mercaptoethanol (Eastman). PMN were isolated from acid-citrate-dextrose anticoagulated blood using a dextran separation method (16). Blood samples were preparatively depleted of platelet-rich plasma by centrifugation at 800 × *g* for 15 min at room temperature and of mononuclear cells by differential centrifugation over a Ficoll-Hypaque gradient (Sigma). After hypotonic lysis of erythrocytes, PMN were washed twice in phosphate-buffered saline (PBS), pH 7.2, and resuspended in ice-cold serum-free RPMI 1640 at 1.5 × 10^7/ml. Suspensions of gel-filtered platelets were prepared as described previously in detail (14).

Antibodies—The establishment and characterization of the se-

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[1] The abbreviations used are: PMN, polymorphonuclear leukocytes; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

quence-specific rabbit polyclonal antibody directed against the human fibrinogen γ chain Lys^{95}-Met^{264} (γ^{95-264}) have been described previously in detail (18). The monoclonal antibody (mAb) 108.3 recognizing an unidentified epitope in the fibrinogen γ chain was the generous gift of Dr. Howard Soule, Corvas International, San Diego, CA. Anti-CD11b mAbs were OKM1 and OKM10 (3). The anti-tissue factor mAb 6B4 (generously provided by Dr. W. Ruf, The Scripps Research Institute), and the anti-factor V rabbit polyclonal antibody V82 were used as controls in antibody inhibition experiments.

Protein Characterization and Labeling—The experimental procedures for the isolation of fibronectin-depleted plasma fibrinogen or D_{30} have been described previously in detail (16). Both fibrinogen and D_{30} were ^{125}I-labeled using the IODO-GEN method (19) to a specific activity of 0.5 and 0.55 μCi/μg, respectively. A panel of synthetic peptides duplicating partially overlapping regions in the fibrinogen γ chain was synthesized and purified to homogeneity by high pressure liquid chromatography on C-18 columns (see Table I). A variant peptide duplicating the fibrinogen γ chain sequence Trp^{191}-Val^{202} was synthesized with the addition of the residues Lys-Tyr-Gly^{190} at the NH_2 terminus for iodination and binding studies. Typically, 2 mg of this peptide, designated P1 (Table I), were iodinated with 5–7 mCi $Na^{125}I$ by the IODO-GEN method for 45 min at 4 °C. Free radioactivity was separated by gel filtration over a Bio-Gel P-2 column (Bio-Rad) preequilibrated with PBS, pH 7.2, with a flow rate of 0.2 ml/s. The factor X-derived synthetic peptides GYDTKQED(G) (366–373), IDRSMKTRG (422–430), and GLYQAKRFKV(G) (238–246) were previously characterized for their ability to inhibit ^{125}I-factor X binding to CD11b/CD18 on chemoattractant-stimulated monocytes or PMN (20, 21).

Binding Reactions—Binding of ^{125}I-fibrinogen or ^{125}I-D_{30} to PMN or THP-1 cells was carried out as described previously (10, 16). Briefly, serum-free suspensions of PMN or THP-1 cells at 1.5×10^7/ml were stimulated with 10 μM of the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma) in the presence of 2.5 mM $CaCl_2$ and 100 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) and mixed with increasing concentrations of ^{125}I-fibrinogen or ^{125}I-D_{30} for 20 min at 22 °C. The reaction was terminated by centrifugation of aliquots of the incubation reaction through mixture of silicone oil (Dow Corning Inc., New Bedford, MA) at $14,000 \times g$ for 5 min to separate free from cell-associated radioactivity. Nonspecific binding was assessed in the presence of a 100-fold molar excess of the corresponding unlabeled competing protein simultaneously added at the start of the incubation reaction and was subtracted from the total to calculate net specific binding. For competition experiments, suspensions of PMN or THP-1 cells were separately preincubated with various doses of the fibrinogen-derived or factor X-derived synthetic peptides (20, 21) for 30 min at 22 °C. Cells were stimulated with 10 μM fMLP in the presence of 2.5 mM $CaCl_2$, 100 μM PPACK, and mixed with subsaturating concentrations of ^{125}I-fibrinogen (0.14 μM) or ^{125}I-D_{30} (0.16 μM) for an additional 20 min of incubation at 22 °C before quantitation of specific binding.

Binding of ^{125}I-fibrinogen to ADP-stimulated gel-filtered platelets in the presence or in the absence of various fibrinogen-derived synthetic peptides or control peptide GRGDSP was quantitated as described previously in detail (14).

For time course studies of ^{125}I-P1 peptide binding, serum-free suspensions of THP-1 cells were incubated with a fixed concentration of the radioiodinated peptide in the presence of 2.5 mM $CaCl_2$ for increasing time intervals between 5 and 60 min at 22 °C. Specific binding was calculated at each time point in the presence of a molar excess (500 μM) of unlabeled P1 or control peptide L10, derived from the COOH terminus of the fibrinogen γ chain (Table I), simultaneously added at the start of the incubation. In another series of experiments, suspensions of THP-1 cells were stimulated with 10 μM fMLP in the presence of 2.5 mM $CaCl_2$ and simultaneously mixed with a fixed dose of ^{125}I-P1 peptide and unlabeled fibrinogen (0.44 μM), unlabeled D_{30} (3.3 μM), or myoglobin (3.3 μM). After 30 min of incubation at 22 °C, specific ^{125}I-P1 binding in the presence of the various competing proteins was quantitated as described.

Binding Reactions to Isolated CD11b/CD18—The recognition of CD11b/CD18 for the P1 sequence was further characterized in a cell-free system. For these experiments, a capture-antibody method was developed to immuno-isolate CD11b/CD18 from detergent-solubilized THP-1 cell extracts as described previously (8). Briefly, 96-well enzyme-linked immunosorbent assay microtiter plates (Falcon) were coated with 10 μg/ml anti-CD11b mAb OKM10 in 0.1 mM $NaHCO_3$, pH 9.0, for 12 h at 4 °C. THP-1 cells (10^9 cells) were solubilized in a lysis buffer containing 0.5% Triton X-100 (Sigma) and 0.5% Nonidet P-40 (Sigma), 1 mM benzamidine (Calbiochem), 100 μM PPACK, 25 μg/ml leupeptin (Calbiochem), 1 mM phenylmethanesulfonyl fluoride (Sigma), and 100 μg/ml soybean trypsin inhibitor (Sigma) for 30 min at 4 °C in constant agitation. Nuclei and other detergent-insoluble materials were cleared by centrifugation at $14,000 \times g$ for 15 min at 4 °C. One hundred-μl aliquots of the cell extract were incubated on OKM10-coated plates for 18 h at 4 °C. Wells were extensively washed in PBS, pH 7.2, post-coated with 3% gelatin (Sigma) for 30 min at 37 °C, and finally incubated in duplicate samples with 0.14 μM ^{125}I-fibrinogen in serum-free RPMI 1640 plus 2.5 mM $CaCl_2$ or 1 mM $MnCl_2$ for 45 min at 22 °C. The reaction was terminated by three washes in serum-free RPMI 1640, and radioactivity associated under the various conditions was quantitated in a γ counter. Nonspecific binding was assessed in the presence of a 100-fold molar excess of unlabeled fibrinogen added at the start of the incubation and was subtracted from the total to calculate net specific binding. The effect of various synthetic peptides on ^{125}I-fibrinogen binding to isolated CD11b/CD18 was assessed by mixing increasing concentrations of control L10 peptide or P1 peptide (15–260 μM) simultaneously with 0.14 μM ^{125}I-fibrinogen for 45 min at 22 °C before quantitation of specific binding as described above.

Adhesion Assays—For these experiments 96-well polystyrene microtiter plates (Lincoln Scientific) were coated with 5 μg/ml fibronectin or fibronectin-depleted fibrinogen for 16 h at 4 °C. At the end of the incubation, wells were rinsed in PBS, pH 7.2, and post-coated with 3% gelatin for 30 min at 37 °C. One hundred-μl aliquots of fMLP-stimulated PMN or THP-1 cells at 5×10^6/ml were equilibrated with various doses of control L10 or P1 peptide (30–500 μM) for 30 min at 22 °C, and subsequently added to the immobilized substrates for 30 min at 22 °C in the presence of 2.5 mM $CaCl_2$. Nonadherent or loosely adherent cells were removed by three washes with serum-free RPMI 1640, and specifically attached cells were stained with 0.5% crystal violet (Sigma) for 10 min at 22 °C. After washing with PBS, pH 7.2, cells were lysed in 0.1% Triton X-100, and the absorbance ($OD_{600\,nm}$) was quantitated on a Vmax Microdevice 96-well plate enzyme-linked immunosorbent assay reader. Alternatively, serum-free THP-1 cells at 5×10^6/ml were metabolically labeled with 0.5 mCi ^{51}Cr (Na_2CrO_4, specific activity, 487.4 mCi/mg, Du Pont-New England Nuclear) for 2 h at 37 °C to a specific activity of 3–5 cpm/cell. After two washes in serum-free RPMI 1640, labeled cells were equilibrated with various doses of control L10 or P1 peptide as described above, plated on the various immobilized substrate for the indicated time, washed, and finally lysed in 20% SDS. Radioactivity was measured in a scintillation β counter. The number of specifically attached cells was calculated by dividing the counts/min harvested by the counts/min per cell.

In another series of experiments, 96-well polystyrene plates were coated with 10 μg/ml of various fibrinogen peptides in 0.1 M $NaHCO_3$, pH 9.0, for 18 h at 4 °C, post-coated with 3% gelatin, and washed in PBS, pH 7.2. Wells were further incubated with 100-μl aliquots of serum-free suspensions of fMLP-stimulated ^{51}Cr-labeled THP-1 cells (1×10^7/ml) in the presence of 2.5 mM $CaCl_2$ for 45 min at 22 °C. After three washes in serum-free RPMI 1640, specifically attached cells were quantitated as described above, or alternatively, by direct analysis of selected fields using a Nikon Diaphot TMD (Nippon Kogaku Co., Japan) inverted microscope. The efficiency of peptide immobilization on plastic wells under these experimental conditions was quantitated experimentally. For these experiments, 1:200 dilutions of a rabbit anti-fibrinogen polyclonal antibody (18) were incubated with the various immobilized peptides for 2 h at 37 °C. After washes in PBS, pH 7.2, binding of the anti-fibrinogen antibody to control peptides or to the various fibrinogen-derived peptides was quantitated by addition of 5 μg/ml aliquots of ^{125}I-labeled goat anti-rabbit antibody for 2 h at 37 °C. Radioactivity specifically associated under the various conditions tested was quantitated in a γ counter.

To assess the specificity of the peptide-mediated adhesion, aliquots of ^{51}Cr-labeled THP-1 cells were incubated with 50 μg/ml anti-CD11b mAb OKM1 for 30 min at 22 °C before reconstitution of the adhesion assay as described above.

RESULTS

Structure-Function Analysis of D_{30}—Initial insights into the identity of the fibrinogen chain remnants in D_{30} mediating binding to CD11b/CD18 (16) were obtained using existing antibodies to the γ chain. For these studies, increasing concentrations of a rabbit polyclonal antibody directed against γ

95–264 (anti-γ^{95-264}) (18), inhibited the binding of ^{125}I-D_{30} to chemoattractant (fMLP)-stimulated PMN in a dose-dependent manner. The reaction was completely abrogated at a 1:100 dilution of antiserum, whereas a control antiserum was without effect under the same experimental conditions (Fig. 1). Similar results were also obtained using the anti-γ chain mAb 108.3 (Fig. 1). Both anti-γ^{95-264} antibody and mAb 108.3 also inhibited ^{125}I-fibrinogen binding to fMLP-stimulated PMN in a dose-dependent manner (not shown).

Peptidyl Mimicry of the Fibrinogen $\gamma^{190-202}$ Chain—To directly investigate whether a recognition sequence for CD11b/CD18 resided in the fibrinogen γ^{95-264} region, peptidyl mimicry experiments were carried out. Partially overlapping synthetic peptides duplicating various aspects of this implicated region of the γ chain (Table I) were initially tested for their ability to inhibit binding of ^{125}I-fibrinogen to stimulated THP-1 cells. In these experiments, increasing concentrations (3–300 μM) of P1 peptide (Gly^{190}-Val^{202} with Lys-Tyr residues at the NH_2 terminus) blocked binding of ^{125}I-fibrinogen to fMLP-stimulated THP-1 cells in a dose-dependent manner (Fig. 2). Maximal inhibition (IC_{50} = 4–8 μM) required preincubation of THP-1 cells with P1 for 30 min at 22 °C (see below). At concentrations >300 μM, P1 aggregated and caused nonspecific precipitation of ^{125}I-labeled fibrinogen. Under the experimental conditions described above, a control peptide synthesized with a scrambled sequence (L-10-Y), or the L10 peptide, duplicating the COOH terminus of the fibrinogen γ chain (Table I) were completely ineffective (Fig. 2). With the exception of DII and P1b peptides (Table I), that partially inhibited ^{125}I-fibrinogen binding to fMLP-stimulated THP-1 cells (see below), none of the other peptides listed in Table I significantly reduced CD11b/CD18 interaction with fibrinogen or D_{30} when tested at concentrations ranging between 50 and 500 μM (not shown).

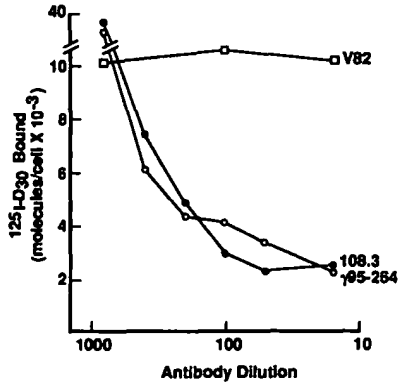

FIG. 1. **Effect of anti-fibrinogen γ chain antibodies on ^{125}I-D_{30} binding to PMN.** Suspensions of dextran-isolated PMN at 1.5 × 10^7/ml were stimulated with 10 μM fMLP in the presence of 2.5 mM $CaCl_2$, and immediately mixed with 0.16 μM ^{125}I-D_{30}, which had been preincubated for 45 min at 22 °C with increasing concentrations of anti-γ chain mAb 108.3, anti-γ chain antibody γ^{95-264}, or control antibody V82. After 20-min incubation at 22 °C, the reaction was terminated by centrifugation through silicone oil at 12,000 × *g* for 5 min at room temperature. Nonspecific binding, ranging between 5 and 15%, was quantitated in the presence of a 50-fold molar excess unlabeled D_{30}, and was subtracted from the total to calculate net specific binding. Shown in the figure is a representative experiment of at least three independent determinations. Similar results were also obtained using monocytic THP-1 cells (not shown in the figure).

TABLE I

Synthetic fibrinogen γ chain and related peptides used in this study

Synthetic peptides were purified by high pressure liquid chromatography on C18 columns. Amino acid sequences are reported in single letter code.

Synthetic peptide	Sequence[a]
γ^1	M^{89}LEEIMKYEASILTHDS^{105}C*
γ^2	H^{103}DSSIRYLQEIYNSNNQ^{119}C*
γ^3	N^{117}NQKIVNLKEKVAQLEA^{133}C*
γ^4	L^{131}EAQCQEPCKDTVQIHD^{147}C*
γ^5	I^{145}HDITGKDCQDIANKGA^{161}C*
γ^6	G^{165}LYFIKPLKANQQFLVYCEIDGSG^{188}C*
DI	I^{169}KPLKANQQFLVYCEIDGSGNG^{190}
DII	Q^{195}KRLDGSVDFKK^{206}
DIII	K^{162}QSGLYFIKPLKAN^{175}
DIV	D^{185}GSGNGWTVFQKR^{197}
P1	K*Y*G^{190}WTVFQKRLDGSV^{202}
P1b	K*Y*G*Q^{195}KRLDGS^{201}
L-10-Y	LGSDKQKRGY
P1′	K*Y*G^{190}WTVFQKRL-GSV^{201}
L10	L^{402}GGAKQAGDV^{411}

[a] * = residues added to the natural sequence; - = deletion.

In parallel experiments, increasing concentrations of P1 caused a dose-dependent inhibition (IC_{50} = 70–80 μM) of the adhesion of fMLP-stimulated THP-1 cells to immobilized fibrinogen (Fig. 3). Consistent with the specificity of CD11b/CD18 recognition of fibrinogen (8, 10–13), concentrations of P1 up to 500 μM did not interfere with attachment of THP-1 cells to fibronectin-coated plates under similar experimental conditions ($OD_{600\,nm}$ P1 = 1.2; L-10-Y = 0.90).

Interaction of P1 with CD11b/CD18—To distinguish between genuine peptidyl mimicry and other effects on receptor function, aliquots of P1 were ^{125}I-labeled and their interaction with THP-1 cells analyzed. As shown in Fig. 4, ^{125}I-P1 associated with fMLP-stimulated THP-1 cells in a specific and time-dependent reaction, approaching apparent equilibrium after a 30-min incubation at 22 °C (Fig. 4). Binding of ^{125}I-P1 to THP-1 cells was maximal in the presence of 1 mM $MnCl_2$, was inhibited by excess unlabeled peptide, and was unaffected by the control peptide L10 (Fig. 4). Finally, the CD11b/CD18 ligands, fibrinogen or D_{30} (10, 16), also inhibited ^{125}I-P1 binding to THP-1 cells while a control protein, myoglobin, was ineffective under the same experimental conditions (Fig. 5).

To further establish the recognition specificity of P1, peptide competition experiments were carried out in a cell-free system using isolated CD11b/CD18. For these studies,

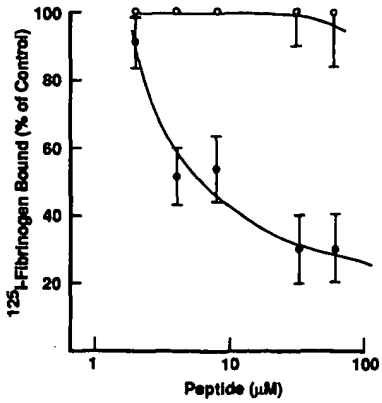

FIG. 2. **Effect of P1 ($\gamma^{190-202}$) on ^{125}I-fibrinogen binding to leukocytes.** Serum-free suspensions of THP-1 cells at 1.5 × 10^7/ml were incubated with increasing concentrations (2–66 μM) of P1 (●) or control peptide L10 (○) in the presence of 2.5 mM $CaCl_2$ for 30 min at 22 °C. Cells were stimulated with 10 μM fMLP and immediately mixed with 0.14 μM ^{125}I-fibrinogen for 20 min at 22 °C. At the end of the incubation, specific binding was calculated as described in Fig. 1. Data are presented as mean ± S.E. of five independent experiments. ^{125}I-Fibrinogen bound to fMLP-stimulated THP-1 cells in the absence of competitors was 40,400 ± 7,900 molecules/cell (n = 6). Similar results (not shown in the figure) were also obtained with fMLP-stimulated PMN.

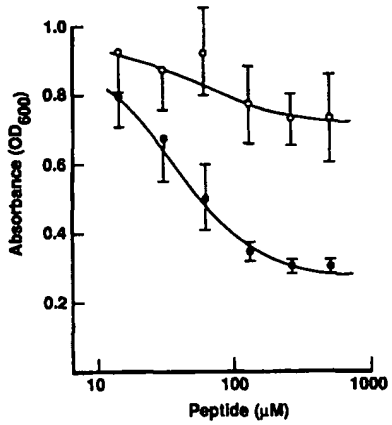

FIG. 3. **Effect of P1 on leukocyte adhesion to fibrinogen.** Aliquots of fibronectin-depleted fibrinogen at 5 μg/ml were immobilized on 96-well polystyrene microtiter wells for 16 h at 4 °C. Plates were washed in PBS, pH 7.2, post-coated with 3% gelatin, and further incubated with serum-free suspensions of fMLP (10 μM)-stimulated THP-1 cells or PMN at 5×10^6/ml for 45 min at 22 °C. In peptide inhibition experiments, aliquots of the leukocyte suspensions were equilibrated with increasing concentrations (15–520 μM) of P1 (●) or L10 (○) in the presence of 2.5 mM $CaCl_2$ for 30 min at 22 °C, before addition to the fibrinogen-coated plates. At the end of the incubation, nonadherent cells were removed by three gentle washes with PBS, pH 7.2, stained with 0.5% crystal violet, lysed in 0.1% Triton X-100, and the absorbance quantitated at $OD_{600\,nm}$. Data are expressed as mean ± S.E. of six indepedent experiments using THP-1 cells. Basal level of THP-1 cell adhesion to fibrinogen-coated plates in the absence of competitors was $OD_{600\,nm}$= 1.076 ± 0.28 (n = 6). Similar results (not shown in the figure) were obtained with fMLP-stimulated PMN.

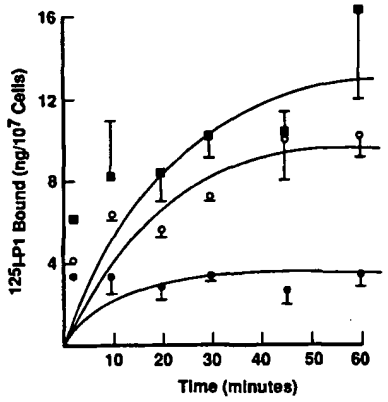

FIG. 4. **Binding of ^{125}I-P1 to THP-1 cells.** Aliquots of ^{125}I-P1 were mixed with suspensions of THP-1 cells at 1.5×10^7/ml in the presence of 1 mM $MnCl_2$ for increasing time intervals at 22 °C. At the end of each incubation, cell-associated radioactivity was determined as described in Fig. 1. Binding of ^{125}I-P1 was assessed in the absence of competing peptides (○), or in the presence of 500 μM unlabeled P1 (●), or L10 (■). Data are presented as mean ± S.E. of two independent experiments.

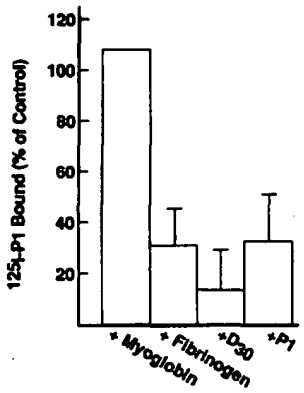

FIG. 5. **Effect of CD11b/CD18 macromolecular ligands on ^{125}I-P1 binding to THP-1 cells.** The experimental conditions are the same as in Fig. 4. For competition experiments, 0.44 μM fibrinogen, 3.3 μM D_{30}, or 3.3 μM myoglobin, were added to THP-1 cells incubated with ^{125}I-P1 and in the presence of 1 mM $MnCl_2$ for 30 min at 22 °C. Data are expressed as mean ± S.E. of two independent determinations.

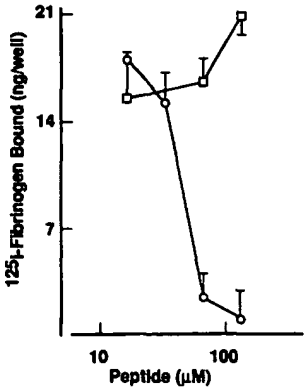

FIG. 6. **Effect of P1 on ^{125}I-fibrinogen binding to immunocaptured CD11b/CD18.** CD11b/CD18 was isolated from detergent-solubilized THP-1 cell extracts (10^9 cells) using OKM10 anti-CD11b mAb immobilized onto 96-well microtiter wells at 10 μg/ml. Wells were preincubated with increasing concentrations (15–260 μm) of P1 (○), or L10 (□) for 30 min at 22 °C in the presence of 1 mM $MnCl_2$ and further incubated with 0.14 μM ^{125}I-fibrinogen. After 45 min at 22 °C, wells were washed three times in serum-free RPMI 1640, amputated, and radioactivity was measured in a γ counter. Specific binding was determined in the presence of a 50-fold molar excess unlabeled fibrinogen. ^{125}I-fibrinogen specifically bound to isolated CD11b/CD18 in the absence of competitors was 23.6 ± 9.1 ng/well. Data are presented as mean ± S.E. of two independent experiments.

CD11b/CD18 was immuno-captured from detergent-solubilized THP-1 cell extracts using OKM10, an anti-CD11b mAb that does not block fibrinogen binding to leukocytes (10, 13). Under these experimental conditions, increasing doses of P1 dose-dependently inhibited binding of ^{125}I-fibrinogen to isolated CD11b/CD18, while control peptide L10 had no effect (Fig. 6).

Specificity of P1 for CD11b/CD18:Fibrinogen Interaction—The possibility that the P1 sequence in the fibrinogen γ chain might function as a unique interacting motif in leukocyte:fibrinogen interaction was further investigated. First, increasing concentrations of P1, that completely inhibited ^{125}I-fibrinogen association with fMLP-stimulated leukocytes (Fig. 2), did not reduce binding of ^{125}I-fibrinogen to ADP-stimulated gel-filtered platelets (Table II). Under these experimental conditions, ^{125}I-fibrinogen binding to stimulated platelets was completely abolished by control peptide GRGDSP, in agreement with previous observations (14) (Table II). Second, binding of ^{125}I-fibrinogen to CD11b/CD18 was only minimally affected (~20–30% reduction) for high concentrations of one (IDRSMKTRG) of the three inhibitory synthetic peptides that completely block ^{125}I-factor X association with CD11b/CD18 on stimulated monocytes (20, 21) (Fig. 7). Finally, analysis of a panel of shorter peptidyl variants of P1 revealed that a minimal inhibitory core sequence was contained within residues Glu^{195}-Val^{202}. As shown in Fig. 8, the corresponding peptide, P1b (Table I), inhibited ^{125}I-fibrinogen binding to fMLP-stimulated THP-1 cells, while the control scrambled peptide L-10-Y was ineffective under the same experimental conditions (Fig. 8). Furthermore, mu-

TABLE II

Effect of various fibrinogen-derived synthetic peptides on ^{125}I-fibrinogen binding to ADP-stimulated gel-filtered platelets

Aliquots of gel-filtered platelets at 1 × 10^8/ml in Tyrode's buffer, pH 7.4, were stimulated with 10 μM ADP and immediately mixed with 0.3 μM ^{125}I-fibrinogen and the indicated peptide concentrations. The reaction was continued for 30 min at 22 °C, before quantitation of specific binding in the presence of a 50-fold molar excess of unlabeled fibrinogen. Data are presented as mean ± S.E. of two independent experiments.

Competitor		^{125}I-fibrinogen bound
	μM	*molecules/platelet*
None		35,000 ± 7,500
GRGDSP	50	10,000 ± 1,700
GRGDSP	100	7,900 ± 370
P1	50	55,400 ± 3,500
P1	100	87,100 ± 6,200
P1b	50	52,300 ± 4,200
P1b	100	67,700 ± 4,200
L-10-Y	50	27,700 ± 940
L-10-Y	100	30,700 ± 1,700

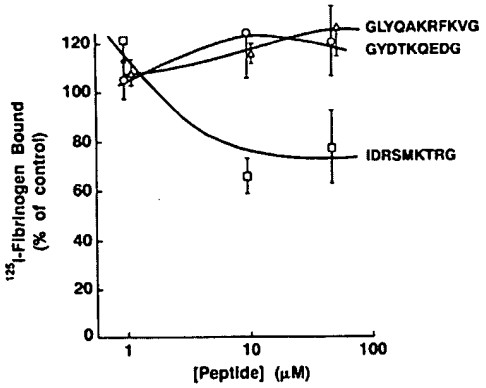

FIG. 7. **Effect of factor X synthetic peptides on ^{125}I-fibrinogen binding to THP-1 cells.** Aliquots of THP-1 cells at 1.5 × 10^7/ml were equilibrated with increasing concentrations of the indicated factor X-derived synthetic peptides for 30 min at 22 °C in the presence of 2.5 mM $CaCl_2$. After stimulation with 10 μM fMLP, THP-1 cells were further incubated with 0.14 μM ^{125}I-fibrinogen for an additional 20 min at 22 °C before quantitation of specific binding. Data are presented as mean ± S.E. of two independent experiments.

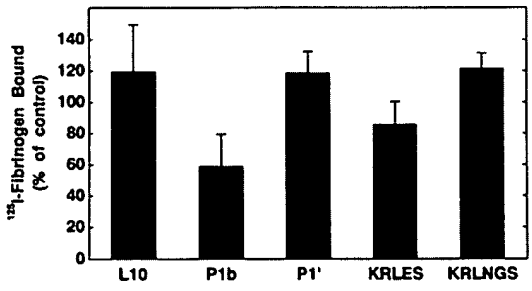

FIG. 8. **Effect of P1 variant peptides on ^{125}I-fibrinogen binding to THP-1 cells.** Aliquots of THP-1 cells at 1.5 × 10^7/ml were equilibrated with 50 μM of the indicated fibrinogen-derived peptides (Table I) for 30 min at 22 °C. After stimulation with 10 μM fMLP, cells were incubated with 0.14 μM ^{125}I-fibrinogen for additional 20 min at 22 °C before quantitation of specific binding as described in Fig. 2. Data are presented as mean ± S.E. of two independent experiments.

tant peptides where Asp[199] was either deleted (P1', Table I), or substituted with Asn or Glu residues failed to reduce ^{125}I-fibrinogen binding to stimulated THP-1 cells (Fig. 8).

Adhesion of CD11b/CD18-bearing Cells to Immobilized P1—Additional experiments explored the possibility that the P1 sequence was in itself sufficient to mediate CD11b/CD18-dependent leukocyte adhesion. To test this hypothesis, microtiter wells were coated with various fibrinogen peptides and incubated with fMLP-stimulated THP-1 cells at 5 × 10^6/ml for 45 min at 22 °C. As shown in Fig. 9, stimulated THP-1 cells bound avidly to immobilized P1, DII peptides (Fig. 9) and P1b (not shown). In contrast, control peptides L10, Gly-Pro-Arg-Pro, L-10-Y (Table I), or RGDS failed to support THP-1 cell adhesion under the same experimental conditions (Fig. 9). As determined experimentally by their reactivity with an anti-fibrinogen antibody (18) in enzyme-linked immunosorbent assay, the amount of these latter peptides immobilized on plastic wells was quantitatively indistinguishable as compared with that of the other functionally active peptides P1, DII, or P1b tested under these experimental conditions (not shown).

To further substantiate the participation of CD11b/CD18 in THP-1 cell attachment to P1, mAb inhibition experiments were carried out. As shown in Table III, saturating concentrations of the functionally inhibitory anti-CD11b mAb OKM1 (10, 12, 13, 16) effectively blocked the adhesion of ^{51}Cr-labeled THP-1 cells to P1-coated plates, while a control mAb was ineffective under the same experimental conditions (Table III).

DISCUSSION

In this study we have identified a novel sequence in fibrinogen that functions as a structural interacting motif for the leukocyte integrin CD11b/CD18. Although originally estab-

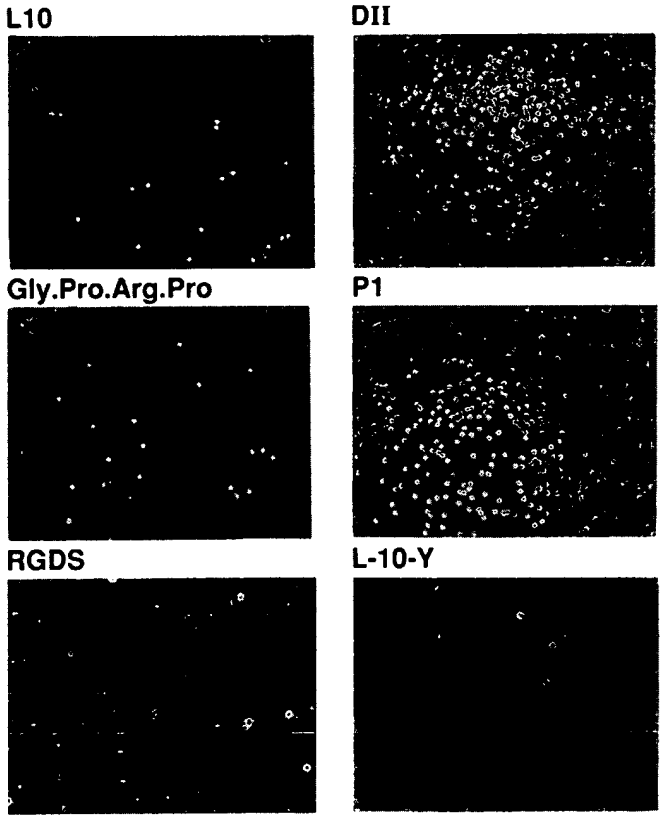

FIG. 9. **Adhesion of THP-1 cells to peptide-coated plates.** 96-well polystyrene microtiter wells were coated with 10 μg/ml of the indicated fibrinogen-derived peptides in 0.1 M $NaHCO_3$, pH 9.0, for 12 h at 4 °C, washed in PBS, pH 7.2, and post-coated with 3% gelatin. Serum-free suspensions of THP-1 cells at 2 × 10^7/ml were added to the various peptide-coated wells for 45 min at 22 °C. After three washes in serum-free RPMI 1640, the degree of cell attachment to the indicated peptides was evaluated microscopically.

TABLE III

Effect of anti-CD11b mAb OKM1 on THP-1 cell attachment to P1-coated plates

Aliquots of ^{51}Cr-labeled THP-1 cells at 5×10^6/ml were incubated with 50 μg/ml anti-CD11b mAb OKM1, or control mAb 6B4 for 30 min at 22 °C. At the end of the incubation, cells were plated on P1-coated plates for 30 min at 22 °C, washed, and lysed in 20% SDS, and radioactivity associated under the various conditions was measured in a β scintillation counter. The number of attached cells was calculated by dividing the counts/min harvested by the counts/min per cell.

mAb	Specificity	Attached cells
None		21,260
6B4	Tissue factor	24,860
OKM1	CD11b	5,070

lished as a complement receptor participating in immune adherence and phagocytosis of opsonized particles (3, 22), recent work has identified CD11b/CD18 as an inducible and high affinity receptor for fibrinogen (10–13). This recognition is mediated by a unique interacting motif contained within a ~30-kDa plasmic fragment D of the ligand (D_{30}). As this fragment of fibrinogen lacks the Arg-Gly-Asp sequences in the Aα chain and the extreme carboxyl terminus of the γ chain (16), CD11b/CD18 recognition of fibrinogen is distinct from that of other vascular integrins (14, 15). Here, a combined strategy based on sequence-specific antibodies and synthetic peptidyl mimicry has converged to identify the fibrinogen $\gamma^{190-202}$ chain as a linear sequence that directly interacts with CD11b/CD18. First, an anti-γ^{95-264} chain antibody (18) inhibited ^{125}I-fibrinogen or ^{125}I-D_{30} binding to stimulated PMN or THP-1 cells. Second, analysis of a panel of partially overlapping γ chain synthetic peptides (Table I) revealed that only the P1 sequence $\gamma^{190-202}$ had specific inhibitory properties on CD11b/CD18 ligand recognition.

P1 inhibited binding of ^{125}I-fibrinogen (IC_{50} = 10–70 μM) to stimulated PMN or THP-1 cells in a dose-dependent fashion, blocked adhesion of these cells to immobilized fibrinogen, and prevented ^{125}I-fibrinogen binding to isolated CD11b/CD18. When tested under the same experimental conditions, none of the other γ chain peptides significantly affected CD11b/CD18 ligand binding. Consistent with genuine peptidyl mimicry, ^{125}I-P1 bound specifically and saturably to leukocytes in a reaction that approached apparent equilibrium after a 30-min incubation at 22 °C, and that was completely abrogated by molar excess fibrinogen or D_{30}. In agreement with previous observations on CD11b/CD18 recognition of macromolecular ligands (8), optimal binding of ^{125}I-P1 to CD11b/CD18 was observed in the presence of mM doses of Mn^{2+} ions (8).

Several lines of evidence indicate that the fibrinogen:leukocyte interaction mediated by CD11b/CD18 has important pathophysiologic implications. Intravascular leukocyte accumulation severely contributes to the onset and development of the atherothrombotic lesion, with disruption of the endothelial cell monolayer, foam cell formation, and release of vasoactive mediators (23–26). Local activation of coagulation and fibrin(ogen) deposition on the leukocyte surface invariably contributes to thrombus organization in the atherosclerotic plaque (27–30). On the other hand, recent studies have provided evidence of how fibrinogen binding to leukocyte CD11b/CD18 also participates in specific mechanisms of inflammation and host defense. In this context, occupancy of CD11b/CD18 with fibrinogen or with the functionally blocking mAb OKM1, profoundly modulates monocyte oxidative responses and Fc-mediated phagocytosis of opsonized particles (31), synergizes with suboptimal doses of cytokine to stimulate large generation of hydrogen peroxide in PMN (32), and initiates monocyte translation of early activation-dependent genes in combination with substimulatory doses of bacterial lypopolysaccharide (33).

The identification of the P1 sequence may be an important step in the development of highly specific probes and antagonists of CD11b/CD18:fibrinogen interaction and its consequences. As characterized in this study, P1 possesses a unique specificity to selectively block fibrinogen association with leukocyte CD11b/CD18, without affecting receptor function of other vascular integrins. In this context, inhibitory doses of P1 did not reduce binding of ^{125}I-fibrinogen to ADP-stimulated gel-filtered platelets (14), and similarly, synthetic antagonists of factor X association with CD11b/CD18 (20, 21) were ineffective in blocking ^{125}I-fibrinogen binding to fMLP-stimulated THP-1 cells.

Two general conclusions can be drawn from these studies. First, the structural basis of fibrinogen association with CD11b/CD18 is not mediated through an Arg-Gly-Asp-dependent recognition. As postulated earlier by analysis of D_{30} binding to CD11b/CD18 (16), we have now identified the sequence $\gamma^{190-202}$ as a novel fibrinogen interacting motif with CD11b/CD18. When this was further dissected in additional experiments using shorter P1 peptide variants, it was found that the P1b peptide, Gln^{195}-Val^{201} inhibited ^{125}I-fibrinogen binding to stimulated CD11b/CD18. However, the inhibitory activity of P1b was decreased approximately 10-fold as compared with that of the longer P1 sequence, with IC_{50} = ~60 μM. The presence of an Asp at position 199 in the inhibitory sequences of P1 and P1b is consistent with the presence of an Asp in most ligand peptides interacting with integrins (1–5). In agreement with its potential involvement in ligand binding suggested for other integrins (1–2), P1 variants, where Asp^{199} was either deleted (P1′, Table I) or mutated to Glu or Asn (Fig. 8), failed to inhibit ^{125}I-fibrinogen binding to CD11b/CD18.

Second, this $\gamma^{190-202}$ chain sequence appears to contain a minimal structural requirement for genuine ligand mimicry, as judged by its ability not only to inhibit receptor binding properties, but also to directly support leukocyte adhesion. Our studies cannot presently exclude the participation of other lower affinity contact sites within fibrinogen, in addition to the $\gamma^{190-202}$ chain, which contribute to an optimal receptor:ligand docking. Consistent with this possibility, P1-mediated inhibition of ^{125}I-fibrinogen binding to leukocytes, or leukocyte attachment to immobilized fibrinogen consistently ranged between 65–80%. If additional sites were involved, this would be similar to the multipartite ligand recognition characteristic of factor X association with CD11b/CD18 (20, 21), and of fibrinogen interaction with platelet $\alpha_{IIb}\beta_3$ (34).

In summary, we have characterized a discrete region in the fibrinogen γ chain within residues 190–202 that interacts directly with CD11b/CD18, and genuinely mimics the macromolecular ligand. While enforcing the concept that leukocyte integrins use structurally different recognition sequences as compared with Arg-Gly-Asp-directed integrins (1, 2), this work has identified a potential peptidyl antagonist of leukocyte adhesion mechanisms that should not interfere with ligand binding to other vascular cell integrins.

REFERENCES

1. Hynes, R. O. (1992) *Cell* **69**, 11–25
2. Ruoslahti, E. (1991) *J. Clin. Invest* **87**, 1–5
3. Sanchez-Madrid, F., Nagy, J. A., Robbins, E., Simon, P., and Springer, T. A. (1983) *J. Exp. Med.* **158**, 1785–1803
4. Springer, T. A. (1990) *Nature* **346**, 425–434
5. Arnaout, M. A. (1990) *Blood* **75**, 1037–1050
6. Altieri, D. C., Wiltse, W. L., and Edgington, T. S. (1990) *J. Immunol.* **145**, 662–670
7. Detmers, P. A., Lo, S. K., Olsen-Egbert, E., Walz, A., Baggiolini, M., and Cohn, Z. (1990) *J. Exp. Med.* **171**, 1155–1162

8. Altieri, D. C. (1991) *J. Immunol.* **147,** 1891–1989
9. Dransfield, I., Cabanas, C., Craig, A., and Hogg, N. (1992) *J. Cell Biol.* **116,** 219–226
10. Altieri, D. C., Bader, R., Mannucci, P. M., and Edgington, T. S. (1988) *J. Cell Biol.* **107,** 1893–1900
11. Wright, S. D., Weitz, J. I., Huang, A. J., Levin, S. M., Silverstein, S. C., and Loike, J. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7734–7738
12. Trezzini, C., Jungi, T. W., Kuhnert, P., and Peterhans, E. (1988) *Biochem. Biophys Res. Commun.* **156,** 477–484
13. Gustafson, E. J., Lukasiewicz, H., Wachtfogel, Y. T., Norton, K. J., Schmaier, A. H., Niewiarowski, S., and Colman, R. W. (1989) *J. Cell Biol.* **109,** 377–387
14. Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A., and Ginsberg, M. H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8057–8061
15. Cheresh, D. A., and Spiro, R. C. (1987) *J. Biol. Chem.* **262,** 17703–17711
16. Altieri, D. C., Agbanyo, F., Plescia, J., Ginsberg, M. H., Edgington, T. S., and Plow, E. F. (1990) *J. Biol. Chem.* **265,** 12119–12122
17. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980) *Int. J. Cancer* **26,** 171–176
18. Fair, D. S., Edgington, T. S., and Plow, E. F. (1981) *J. Biol. Chem* **256,** 8018–8023
19. Fraker, P. J., and Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857
20. Altieri, D. C., and Edgington, T. S. (1988) *J. Biol. Chem.* **263,** 7007–1015
21. Altieri, D. C., Etingin, O. R., Fair, D. S., Brunck, T. K., Geltosky, J. E., Hajjar, D. P., and Edgington, T. S. (1991) *Science* **254,** 1200–1202
22. Ezekowitz, R. A. B., Sim, R. B., and Gordon, S. (1983) *J. Exp. Med.* **159,** 244–260
23. Gerrity, R. G. (1981) *Am. J. Pathol.* **103,** 181–190
24. Gerrity, R. G. (1981) *Am. J. Pathol.* **103,** 191–200
25. Aqel, N. M., Ball, R. Y., Waldman, H., and Mitchinson, M. J. (1985) *J. Pathol.* **146,** 197–204
26. Stary, H. C. (1987) *Atherosclerosis* **64,** 91–108
27. Colvin, R. B., and Dvorak, H. F. (1975) *J. Exp. Med.* **142,** 1377–1390
28. Colvin, R. B., Mosesson, M. W., and Dvorak, H. F. (1979) *J. Clin. Invest.* **63,** 1302–1306
29. Sherman, L., and Lee, J. (1977) *J. Exp. Med.* **145,** 76–85
30. Hogg, N. (1983) *J. Exp. Med.* **157,** 473–485
31. Trezzini, C., Schüepp, B., Maly, F. E., and Jungi, T. W. (1991) *Br. J. Haematol.* **77,** 16–24
32. Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J., and Wright, S. D. (1989) *J. Cell Biol.* **109,** 1341–1349
33. Fan, S.-T., and Edgington, T. S. (1991) *J. Clin. Invest.* **87,** 50–57
34. Santoro, S. A., and Lawing, W. J., Jr. (1987) *Cell* **58,** 867–873

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RESTENOSIS AFTER CORONARY ANGIOPLASTY

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RESTENOSIS AFTER CORONARY ANGIOPLASTY

CONTENTS

RESTENOSIS AFTER CORONARY ANGIOPLASTY

ABSTRACT.—Coronary angioplasty is used to treat coronary disease in many patients. Indications for angioplasty have expanded since it was first performed, mainly as a result of improvement in equipment and techniques. One problem with coronary angioplasty is the phenomenon of renarrowing of the treated coronary lesion, a process called *restenosis*. The events that constitute restenosis appear to be a universal response to the arterial wall injury of angioplasty. They are currently characterized as follows: platelet adhesion and aggregation on the damaged endothelium and within deep splits into the tunica media; release of platelet-derived growth factors; inflammation of the mechanically injured medial zone; transformation of smooth muscle cells of the tunica media after their activation by several of the growth-promoting substances; migration and proliferation of transformed smooth muscle cells, with secretion of copious amounts of extracellular matrix material; and, finally, termination of the growth process with regrowth of endothelium over the injured area. A decade of research work has helped identify clinical correlates of restenosis after coronary angioplasty procedures. This work is hindered by lack of a uniform angiographic definition of restenosis. In addition, much of the information has come from small studies, with incomplete follow-up and retrospective orientation. Nevertheless, some data are available. Patient-related correlates include male gender, unstable angina, diabetes, and continued smoking after angioplasty. Lesion-related correlates include multilesional and multivessel procedures, higher postangioplasty residual stenosis, proximal vessel location, location in the left anterior descending artery, location in a vein graft, long le-

FOREWORD

Balloon angioplasty, first used in 1977 when it was hailed as a less-invasive method of improving circulation to the heart muscle, has now been shown to be ineffective in an all-too-high number of cases. The events that cause restenosis of coronary vessels after angioplasty occur after nearly all such surgeries, although the degree of occlusion may vary considerably. The author of this issue of DISEASE-A-MONTH, Dr. H. Vernon Anderson, provides us with a thorough review of the reasons for these postsurgical changes. He also reviews a number of experimental treatments that may minimize restenosis. This is an excellent monograph; I recommend it as an interesting and important piece of writing.

Roger C. Bone, MD
Editor-in-Chief

sions, and total occlusions. The only consistent procedure-related correlate has been incorrect sizing of the angioplasty balloon to the treated artery. For the purposes of individual patient care, clinical correlates are not helpful. No group of variables has been found to be associated with complete freedom from restenosis, and no group is completely predictive of restenosis. All patients undergoing angioplasty procedures require some follow-up through subsequent months and years. Symptom status and the results of noninvasive studies have been investigated for purposes of follow-up. Symptoms are virtually useless by themselves for predicting restenosis or its absence. When symptom status is combined with exercise thallium 201 scintigraphy performed 4 to 6 months after an angioplasty procedure, the two factors are less than ideal but have a negative predictive value of more than 90%. This means that more than 90% of patients who have neither symptoms nor evidence of ischemia by thallium 201 scintigraphy will not have angiographic restenosis. Application of this finding to clinical practice must be tempered by individual patient circumstances, such as whether the patient had symptoms before the angioplasty procedure and whether the potential area of ischemia is even discernible by thallium imaging. Numerous clinical studies have been performed to reduce or prevent restenosis. Almost all have been disappointing, but several have been encouraging. Studies of antiplatelet agents, such as aspirin, dipyridamole, and ticlopidine, have not shown efficacy. On the other hand, studies of an inhibitor of platelet-derived growth factor have been provocatively encouraging. No reduction in restenosis was found with the use of the anticoagulants heparin and coumadin. Fish oils have been found in several trials to provide modest but encouraging reductions in restenosis, but these results await further confirmation. Inhibitors of thromboxane, along with analogs of prostacyclin, have not been found to be effective. A panoply of other agents, including angiotensin-converting enzyme inhibitors, calcium-channel antagonists, β-adrenergic receptor antagonists, lipid-lowering agents, and the antimitotic drug colchicine, have all been found to be ineffective. A number of other mechanical revascularization strategies, including atherectomy procedures and coronary stent devices, can achieve potentially better initial results than standard balloon angioplasty in some situations. Restenosis rates appear to be slightly lower in some groups with these procedures. Further research with these procedures is therefore necessary. Restenosis remains a clinical challenge, but better understanding of coronary artery disease and of its development and treatment will ultimately be derived from studies of the phenomenon of restenosis.

IN BRIEF

Percutaneous transluminal coronary angioplasty is used to treat atherosclerotic coronary obstructions in many patients with stable or unstable angina pectoris, acute myocardial infarction, and asymptomatic ("silent") ischemia. The indications for coronary angioplasty have expanded since its introduction into clinical practice in 1977. Initially, coronary angioplasty was restricted to patients with single-vessel disease and normal left ventricular function. In recent years, many patients with complex, multivessel disease, impaired left ventricular function, or previous bypass surgery have been effectively treated with angioplasty. These changes have come about because of tremendous advances in equipment design, angioplasty techniques, and adjunctive pharmacologic therapies.

One of the problems with angioplasty, which has not changed since its introduction, is the phenomenon of renarrowing *(restenosis)* of the treated coronary artery. Approximately one quarter of initially successful, first-time angioplasty procedures have to be repeated within 6 months, and another 10% or so of patients must later undergo coronary bypass surgery, because of restenosis. Although the clinical problem of restenosis remains as large as ever, new insights into its causes have come from many research studies, clinical as well as basic, during the past decade.

Coronary angioplasty represents deliberate injury to the arterial wall. Apparently, there are four components to this injury: endothelial denudation, breach of the internal elastic lamina, disruption of the tunica media, and stretching of myocytes within the media. The response of the arterial wall to this injury is multifactorial. Elastic recoil of stretched tissues, as well as some reactive vasospasm, can result from the dilating force of an angioplasty balloon. These elements, although probably universal, are not the major processes at work after an angioplasty procedure. More important, after angioplasty injury the arterial wall appears to undergo a healing process characterized by hyperplastic neointimal proliferation. The formation of this neointima occurs with various degrees of severity, which helps explain why recurrent significant obstructive lesions develop in some

but not in others. The arterial wall healing responses after angioplasty appear to center around several processes. One of these is the loss of protective endothelium. Angioplasty is virtually always accompanied by complete endothelial denudation at the treated site. Loss of the endothelium removes antithrombotic and antivasospastic protective mechanisms, promoting adhesion and aggregation of platelets. Platelets attached to the arterial wall at sites of damaged endothelium release several substances that promote the transformation and growth of smooth muscle cells of the tunica media. The action of dilating and stretching an atheromatous artery typically results in splitting and fissuring of the atheroma, rupture of the internal elastic lamina, and tears, splits, and dissections into the tunica media. This damage exposes numerous subendothelial elements to blood components, especially platelets, which have surface receptors for binding many subendothelial elements. Platelets adhering to areas denuded of endothelium or within deep crevices of the media release the contents of their stored granules. These platelet products, including platelet-derived growth factor, serotonin, thrombospondin, and transforming growth factor, promote transformation of smooth muscle cells into a proliferative and secretory phenotype. In addition to the actions of platelet-derived products, stretching of myocytes in the tunica media causes mechanical damage to these cells and their surrounding matrix. This is accompanied by an inflammatory response, with invasion of monocytes, macrophages, and other white blood cells. These inflammatory cells release various cytokines and growth factors that stimulate smooth muscle cells of the media. The end result of these stimulative activities is to induce some normally quiescent, contractile-type smooth muscle cells to undergo a transformation to a proliferative, noncontractile phenotype. These transformed myocytes are similar to fibroblasts. After transformation, some of these cells begin to proliferate, migrate into the intimal area, and produce large quantities of extracellular proteoglycan matrix material. This results in the formation of the neointimal layer that represents the restenotic lesion.

One of the terminating events to neointimal hyperplasia appears to be the regrowth of endothelium over the injured area. When endothelial denudation is not extensive and deep medial injury does not occur, endothelial regrowth is rapid and complete and neointimal hyperplasia is minimal. Conversely, with more extensive endothelial denudation and deeper medial injury, endothelial regrowth is slower and may be incomplete. In this latter situation, neointimal hyperplasia can be quite severe. Endothelial cells at the margins of the zone of injury, which are proliferating and attempting to recover the injury, secrete substances that are inhibitory for migrating and proliferating smooth muscle cells. At the same time, transformed smooth muscle cells that are migrating and proliferating secrete substances

that inhibit endothelial cells. Eventually, a balance of forces is achieved after a variable amount of hyperplastic neointima has formed. Even without complete endothelial regrowth over the injured area, there appears to be a limit to the amount of neointimal hyperplasia that can develop. In chronically denuded areas, intimal hyperplasia peaks at about 8 weeks after injury and then remains stable. This may represent a balanced equilibrium of sorts between the transformed smooth muscle cells and the endothelial cells of the region.

From the clinical perspective, more than a decade of research work has helped to identify clinical correlates of restenosis, as well as to investigate various methods for preventing restenosis. There is some confusion in this field, however. Despite the fact that angiography is the method used to identify significant coronary disease and judge whether angioplasty is successful, there is as yet no uniform angiographic definition of restenosis. Different investigators use different angiographic definitions, and some have used several definitions for the same patient data to demonstrate that restenosis rates can vary widely depending on the particular definition used. Many times, patients with coronary disease are initially detected through the presence of symptoms and the results of noninvasive tests, especially exercise stress tests with or without thallium 201 scintigraphy. However, these tests have been found to be less than ideal for detecting patients with restenosis. Symptoms appear to recur more frequently than do significant coronary obstructions, at least when judged by angiography. The positive predictive value of recurrent symptoms for true restenosis is approximately 67%. This is not changed much by exercise testing protocols with or without thallium 201, which have positive predictive values of approximately 67% and 50%, respectively. Tests performed later after angioplasty (>4 to 6 months) appear to have better predictive values than tests performed earlier. A negative exercise thallium 201 study performed more than 6 months after angioplasty appears to indicate absence of restenosis more than 90% of the time.

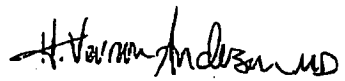
Clinical correlates of restenosis have been identified. They are generally divided into three categories: patient-related factors, lesion-related factors, and procedure-related factors. The patient-related factors include male gender, unstable angina, diabetes, and continued smoking after angioplasty. The lesion-related factors include multilesional and multivessel procedures, higher postangioplasty residual stenosis, proximal vessel lesions, location in the left anterior descending artery, location in a saphenous vein graft, longer lesions, and total occlusions. Finally, the only consistent procedure-related factor found to correlate with restenosis is an incorrect balloon-to-artery diameter ratio (<90% or >115% of the adjacent "normal" artery). The numerous clinical studies that helped to identify these fac-

tors are limited by the aforementioned lack of a uniform definition of restenosis; many also were quite small in size, had incomplete angiographic follow-up, had a variety of individually chosen follow-up intervals, and were entirely retrospective in outlook. These shortcomings help reemphasize the point that, for practical purposes of individual patient care, clinical correlates of restenosis are not helpful. All patients who undergo coronary angioplasty procedures require some form of reevaluation after the procedure. Although the proper follow-up interval and reevaluation strategy have not been determined, for many patients a noninvasive thallium 201 scintigram obtained 4 to 6 months after the procedure appears to be appropriate. Some patients with important coronary disease probably should have coronary arteriograms at 4 to 6 months for their follow-up evaluation.

A number of clinical studies have been performed during the past 10 years to try to reduce or prevent restenosis. Most have been disappointing, although a few have been encouraging. The largest number of studies have investigated the use of various antiplatelet agents to reduce restenosis. The most common antiplatelet agent has been aspirin, followed by dipyridamole and ticlopidine. Almost all of these studies have shown no reduction in restenosis rates, although one study that combined aspirin with ticlopidine and nicorandil (a calcium-channel antagonist), did show substantial benefit. Antiplatelet agents are used currently for their demonstrated benefit of reducing acute complications of angioplasty procedures. Three clinical studies have used an inhibitor of platelet-derived growth factor, trapidil. All three showed large reductions in restenosis rates, but only one of these studies had enough patients to make the decline statistically significant. Further research with trapidil is warranted. Heparin and coumadin, both anticoagulants, have also been investigated. No reduction in restenosis was demonstrated with these agents. Fish oils, usually characterized by the two ω fatty acids eicosapentaenoic acid and docosahexaenoic acid, replace diet-derived linoleic acid as the major prostanoid precursor in the arachidonic acid pathway. The prostanoid products derived biologically from fish oils, thromboxane A_3 and prostaglandin I_3, have less procoagulant and vasoconstrictive activity than the usual products. Clinical trials with fish oils as dietary supplements have been encouraging without being dramatic. Restenosis rates have been reduced fairly consistently, from the 35% to 40% range to the 15% to 20% range. Further research with fish oils is warranted. In contrast to these apparently beneficial effects of fish oils, direct manipulations of prostanoid products have not been effective. Antiaggregatory, antivasospastic prostacyclin-type compounds, as well as inhibitors of thromboxanes, have not reduced restenosis rates. Similarly, two large clinical trials of angiotensin-converting enzyme inhibitors did not demonstrate any benefit. In

some experimental preparations, angiotensin had been shown to be mitogenic toward smooth muscle cells and therefore possibly related to the restenosis process. Calcium-channel antagonists, β-adrenergic receptor antagonists, lipid-lowering agents, and the antimitotic agent colchicine have not been effective against restenosis. Future trials will investigate newer, more specific antiplatelet agents, newer anticoagulant agents, growth-factor inhibitors, and quite possibly gene therapy for the reduction or prevention of restenosis.

Several alternative, or rather complementary, revascularization techniques have been developed in recent years. These include atherectomy, of which there are currently three types of procedures, and coronary stents, of which there are currently five types of devices. Although it was hoped that these alternative or complementary revascularization procedures would reduce rates of restenosis, this has not turned out to be the case. In some studies, the restenosis rates for certain types of lesions do appear to be somewhat lower than they might have been with standard balloon angioplasty, but the differences are small. In specific individual situations, these other devices probably are able to achieve better initial results than standard balloon angioplasty, and a clinical role for them will therefore exist no matter what the restenosis rates are. Restenosis, although a vexing problem clinically, is a fascinating problem biologically. Improved understanding and treatment of atherosclerosis, a disease that causes so much morbidity and mortality in our society, will ultimately be derived from studies of restenosis.



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RESTENOSIS AFTER CORONARY ANGIOPLASTY

Coronary angioplasty is a useful and effective method of revascularization for many patients with atherosclerotic coronary artery disease. Angioplasty is used to treat coronary obstructions in patients with chronic stable angina pectoris, unstable angina, acute myocardial infarction, and asymptomatic or "silent" ischemia. Although it was initially applied only in patients with single, discrete lesions in the proximal portion of one of the major coronary arteries, improvements in equipment and techniques have led to expansion of the application of angioplasty. Many patients with disease in multiple vessels or chronic totally occluded coronary arteries, patients with lesions in saphenous vein grafts, some patients with impaired left ventricular function, and numerous other populations may also be successfully treated. Precise indications for performing coronary angioplasty have changed since the treatment was first introduced in 1977.[1] Rigid criteria cannot currently be specified. In general, both the patient and the physician must be reasonably confident that an angioplasty procedure could reduce a significant atherosclerotic obstruction and relieve or prevent important ischemia, with mutually acceptable risks of morbidity and mortality.

One of the problems with coronary angioplasty as a therapeutic modality is the fact that the treated lesion or lesions may recur. This phenomenon of renarrowing of the coronary lesion to produce significant obstruction again is termed *restenosis*. Although tremendous advances in equipment design, angioplasty techniques, and adjunctive pharmacologic agents have made angioplasty far safer and more effective during the past 15 years, the daunting challenge of restenosis has not yet been surmounted. This monograph outlines and discusses some relevant issues concerning restenosis. The first sections focus on biologic issues that underlie the restenosis process; the later sections focus on clinical issues derived from the biology.

BIOLOGY OF RESTENOSIS

PATHOBIOLOGY OF ARTERIAL INJURY

Coronary angioplasty represents deliberate injury of the arterial wall. The actions of stretching and dilating an eccentric tubular mass of rigid, complex atheroma along with compliant normal arterial wall causes splitting, fissuring, and tearing of the atheroma and wall to various degrees (Fig. 1). In some respects, this is not unlike the process of spontaneous plaque rupture that is responsible for the conversion of stable coronary ischemic syndromes into unstable syndromes.[2,3] Certainly, the vasospasm and thrombosis that occasionally occur after angioplasty mimic circumstances that may occur naturally in unstable ischemic syndromes. Angioplasty injury, however, can be attended by other, more insidious processes not usually found with spontaneous plaque rupture. It is these other processes that constitute the biology of restenosis, which may in fact be a normal (or perhaps abnormally exaggerated) tissue response to the injury of angioplasty. Fig. 2 illustrates the concepts of angioplasty as injury and restenosis as a neointimal proliferative response to this injury. The following sections correspond to the events outlined in Fig. 2. One other process, however, not shown in Fig. 2, probably plays a role in clinical restenosis and perhaps plays the major role in at least some cases. This is the process of elastic recoil of the vessel after being stretched by the angioplasty balloon.

ELASTIC RECOIL

Elastic recoil after coronary angioplasty has been described clinically,[4] and possible mechanisms for this phenomenon, which has elements of vasospasm mixed in, have begun to be investigated experimentally.[5] In a detailed and extensive review of 20 autopsy cases involving patients who died from 1 to 24 months after successful coronary angioplasty, Waller et al.[6] found significant obstruction but no evidence of neointimal proliferation at the angioplasty site in eight cases (40%). This appeared to correspond to their literature review of 41 published autopsy cases, in which approximately 33% of the angioplasty sites did not show evidence of neointimal proliferation, despite being significantly renarrowed. They postulated that elastic recoil of the vessel wall was responsible, and indeed more of the lesions without neointimal proliferation at autopsy were eccentric and therefore had larger amounts of disease-free wall capable of elastic rebound. From a practical standpoint, however, the contribution that elastic recoil makes overall to clinical restenosis is uncertain and is not likely to be meaningful or helpful in treating patients. It is not possible to predict in advance which lesions will undergo elastic recoil only and which are candidates for excessive neointimal proliferation. There do appear to be regional variations in elastic recoil throughout the coronary tree[7] that parallel some of the known correlates for restenosis, but the differences are small and not helpful clinically. For example, the study by Rensing et al.[7] found that asymmetric lesions had significantly more recoil than symmetric lesions,

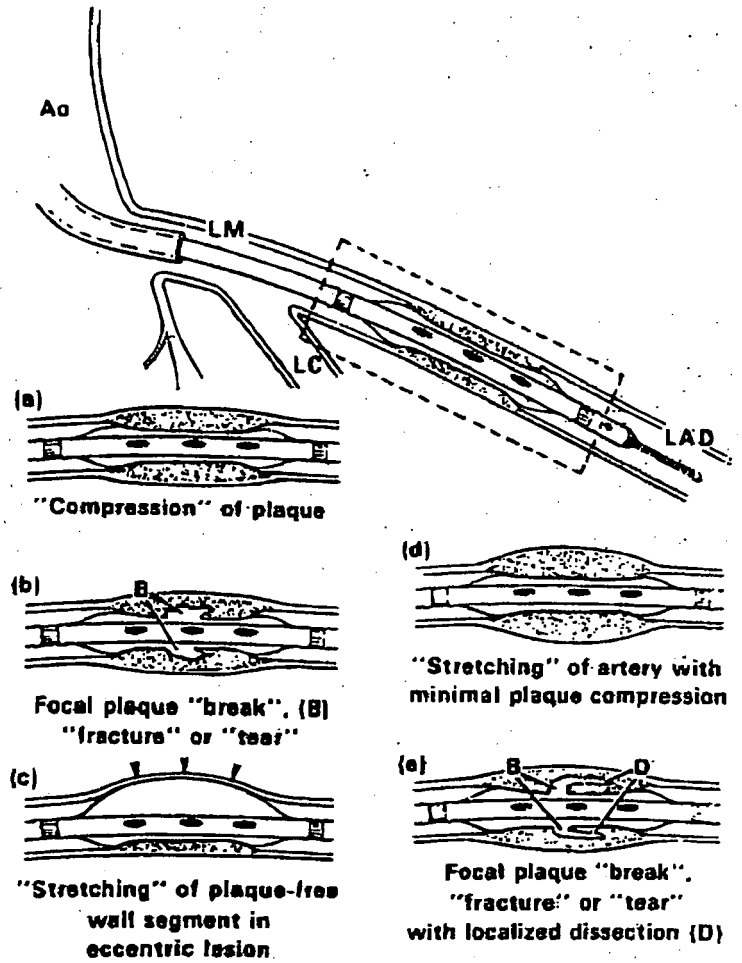


FIG. 1. Schematic diagram illustrating possible actions of coronary angioplasty. *Ao*, Aorta; *LM*, left main coronary artery; *LC*, left circumflex coronary artery; *LAD*, left anterior descending coronary artery. (Reprinted with permission from the American College of Cardiology. Waller BF. Coronary luminal shape and the arc of disease-free wall: morphologic observations and clinical relevance. J Am Coll Cardiol 1985;6:1100–1.)

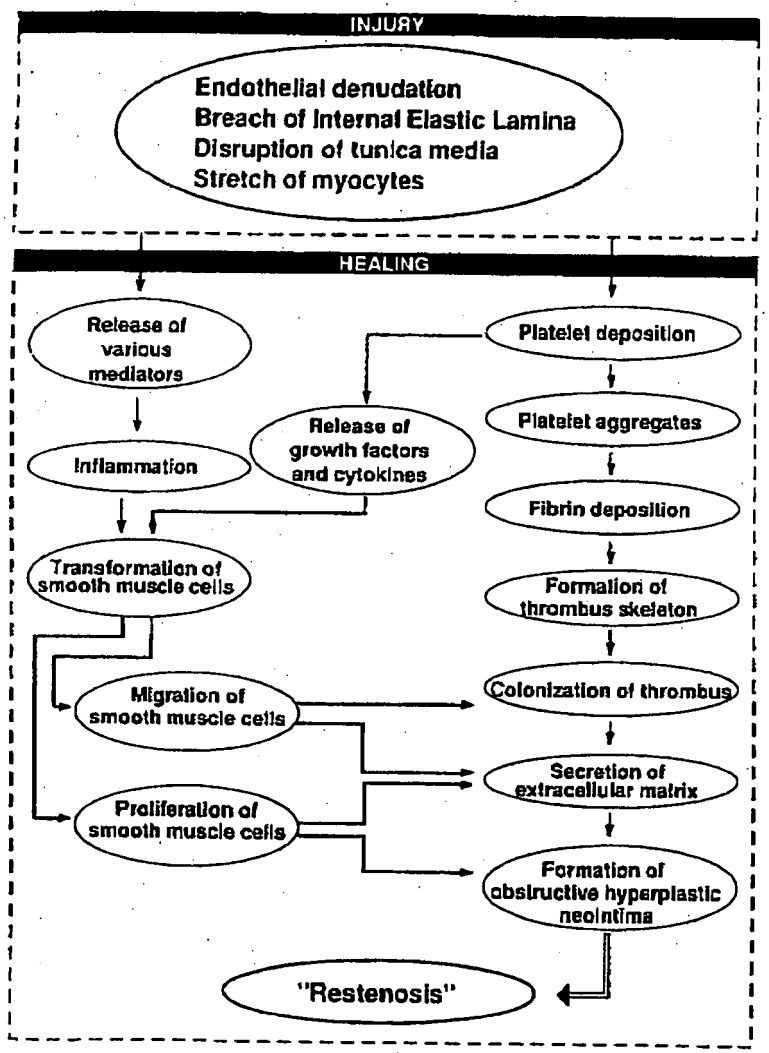


FIG. 2. Schematic diagram illustrating concept of angioplasty as injury and the steps involved in the healing response to angioplasty. The processes involved can result ultimately in the formation of a hyperplastic neointima. Formation of this neointima may recreate an obstructive coronary lesion at the treated site. This is termed *restenosis*.

but the difference (50% recoil vs 43% recoil, $p < 0.05$) would not change treatment for asymmetric lesions in general. The same would be true for the proximal to distal variation in elastic recoil found by Rensing et al.[7] The differences (43% vs 61% recoil for proximal vs distal left anterior descending coronary lesions), although important, are not clinically useful. It would be potentially misleading to think that clinical restenosis rates could be reduced by one third or more if only elastic recoil and vasospasm could be completely prevented. Even though elastic recoil almost certainly occurs in every angioplasty and may even be the only arterial response leading to restenosis in a few cases, it is not predictable and reliable enough, nor preventable enough, to warrant neglecting the hyperplastic neointimal proliferative response that also occurs.

INJURY AND RESPONSE

Two elegant paradigms of restenosis as a response to injury have been proposed by Forrester et al.[8] and Schwartz et al.[9] These two paradigms are not mutually exclusive, and in fact contain numerous overlapping ideas. Together, they form the core concepts of our current understanding of the biology of restenosis. The injurious events detailed for coronary angioplasty include the following (Fig. 2): endothelial denudation, breach of the internal elastic lamina (IEL), disruption of the tunica media, and stretching of myocytes within the media. This injury is then followed by arterial wall healing responses that include platelet deposition, platelet aggregation, accumulation of fibrin onto aggregated platelets to form a thrombus of variable size, release of platelet-derived substances that promote transformation and growth of smooth muscle cells, and release of both platelet-derived and myocyte-derived mediators that engender an inflammatory response (which also promotes transformation and growth of smooth muscle cells). Transformation is followed by migration of some transformed smooth muscle cells from the media into the intimal layers, colonization of the thrombus "skeleton" by migrating smooth muscle cells, proliferation of some migrating smooth muscle cells, production and secretion of extracellular matrix substance by transformed smooth muscle cells, and finally the completion of the proliferative process with reestablishment of equilibrium after (in most cases) the reendothelialization of the luminal surface of the vessel (Fig. 3). Each of these points is discussed as follows.

Endothelial Denudation

Vascular endothelium has numerous functions. Two of these are an antithrombotic function and a vasodilator (or perhaps antivasospastic) function. Normal endothelium secretes substances, such as

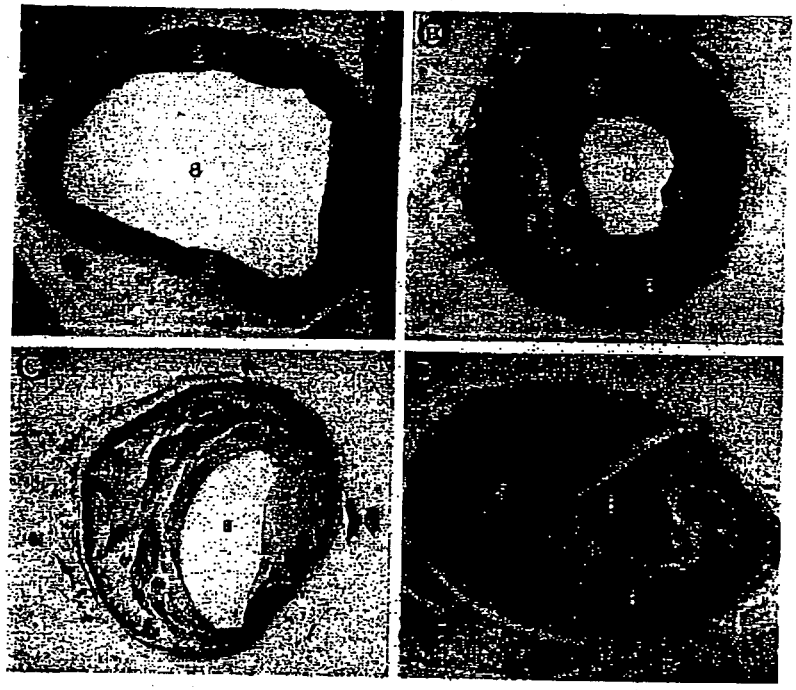


FIG. 3. Photomicrographs of cross-sections of human coronary arteries. **A,** Normal coronary artery has a widely patent, unobstructed lumen. **B,** Coronary artery with advanced native atherosclerosis exhibits luminal narrowing by atherosclerotic plaque. The plaque is composed predominantly of collagen (fibrous tissue) and atheroma (lipid-rich material). **C,** Left anterior descending artery at the site of previous angioplasty. The patient died 2 months after the procedure. There is loss of most of the IEL, destruction of portions of the tunica media, a marked proliferation in the plaque-free segment of the arterial wall, and preservation of the external elastic lamina and tunica adventitia. **D,** Left anterior descending artery at the site of two previous angioplasty procedures, performed 7 months and 5 months earlier. There is severe concentric proliferation narrowing the lumen of the vessel (which contains injected radiographic contrast material). Much of the IEL is missing, and there is destruction of the inner half of the tunica media. Little or no atherosclerotic plaque can be identified in this segment of the artery. *1,* External elastic lamina; *2,* tunica media; *3,* IEL; *4,* collagen; *5,* smooth muscle cell and fibroblast proliferation tissue; *6,* lipid-laden atheroma; *7,* calcification; *8,* vessel lumen. (Original magnifications ×15; **A** and **B,** hematoxylin-eosin stain; **C** and **D,** Verhoeff's van Giesen elastic stain). (**A** and **B** courtesy of Dr. L. M. Buja, University of Texas Health Science Center at Houston; **C** and **D** from: Gravanis MB, Roubin GS. Histopathologic phenomena at the site of percutaneous transluminal coronary angioplasty: the problem of restenosis. Hum Pathol 1989;20:477–85. Used with permission.)

heparin sulfate, thrombomodulin, tissue-type plasminogen activator, and prostacyclin, all of which act to counter prothrombotic and vasospastic influences. Angioplasty performed in experimental animal models is accompanied by almost complete loss of endothelial cells from the luminal surface of the treated arterial segment.[10, 11] Similar endothelial denudation occurs with angioplasty in human patients. Patients dying shortly after coronary angioplasty have been found at postmortem examination to have lost extensive amounts of endothelium at the treated coronary sites.[12, 13] Conversely, it is also known that at least some advanced atherosclerotic plaques lack complete coverage by endothelial cells, and instead appear to have areas covered by modified smooth muscle cells or other cells.[14] Either way, the extensive loss or replacement of endothelial cells, with their protective functions, contributes to the risks for abrupt thrombotic occlusion and coronary vasospasm, both well-documented clinical events in angioplasty, and perhaps even contributes to the risk of restenosis. The reasoning behind this last possibility is the observation that the amount of endothelial denudation appears to correlate, at least in some experimental models, with the degree of later neointimal thickness.[15, 16, 17] Small areas of endothelial loss have been accompanied by extremely little hyperplasia; larger areas of endothelial loss have been accompanied by more extensive hyperplasia. The operative mechanism for this may be the extent of platelet aggregation. Wider areas of endothelial denudation could have more platelet deposition and thus potentially greater amounts of thrombus, whereas smaller areas could have less.

Breach of IEL

As discussed by Schwartz et al.,[18] one of the important natural dividing lines for arterial wall injury may be the integrity of the IEL. Mild injury that only removes endothelial cells from the luminal surface without disrupting the IEL has been shown to be accompanied by little or no neointimal response. More intense injury that breaks the IEL is accompanied by a much more exuberant neointimal response. Because the atheroma is relatively more rigid than any adjacent nonatheromatous arterial wall, breaks and tears are more likely to occur at the junction between the more compliant arterial wall and the atheroma. Whether the IEL represents a true physical barrier that acts to restrain migration and proliferation or whether IEL integrity is only a general marker for injury severity is speculative at present. It is interesting to note, however, that in experimental animal models the most intense neointimal response is found in the regions of the IEL breaks, with a much less intense response in regions remote from the breaks. Pathologic specimens studied by light microscopy as well as by scanning electron microscopy have shown

FIG. 4. Photomicrograph illustrating transformed smooth muscle cell *(SMC)* distorting the IEL as it moves into the neointima *(NEO)*. (Courtesy of Dr. Ward Casscells, University of Texas Health Science Center and Texas Heart Institute, Houston.)

what appear to be transformed smooth muscle cells moving through the IEL as they migrate into the neointima (Fig. 4).

Medial Disruption

With endothelial cell denudation and fracture of the IEL, the tunica media subjacent to the angioplasty site can become fissured and disrupted, exposing numerous subendothelial elements to blood. Angiography at the time of angioplasty is not always sensitive enough to detect this. Although angiography reveals that approximately 25% to 40% of coronary angioplasty sites have evidence of tear and dissection into the medial layers, both Mizuno et al.[19] and Uchida et al.[20] found by means of angioscopy that virtually all angioplasty sites had evidence of subendothelial injury. Pathology studies also have confirmed this finding of more extensive medial injury than what is revealed by standard angiography alone.[21–23] Splitting of the surface of the atheroma exposes amorphous plaque substance (mostly fibro-fatty material) to the bloodstream. Deeper fissures into the tunica media expose collagen, elastin, fibronectin, vitronectin, and other matrix components. Platelets have numerous surface receptors for binding these elements, and deep crevices provide ample opportunities for flow stasis and platelet adhesion. Deeper injury may therefore be a marker for more extensive platelet

accumulation, larger amounts of thrombus, and, as mentioned earlier, breaches of the IEL. Experimental studies suggest that injury that extends more deeply into the arterial wall is accompanied by more intense neointimal proliferation. Ip et al.[24] demonstrated in a pig model that wide but shallow injury engendered little hyperplasia; narrow but deep injury engendered substantially more hyperplasia. Clinically, this conjecture may be supported by observations made with analysis of atherectomy specimens, in which the more medial tissue extracted (deeper injury) the higher the restenosis rate. Such observations have in fact been made by Garratt et al.[25] In contrast, however, a recent larger study by Kuntz et al.[26] did not find any correlation between depth of excision with atherectomy and subsequent restenosis rates.

Myocyte Stretch Injury

Mechanical injury to myocytes, in this case the smooth muscle cells of the tunica media, can stimulate some myocytes to undergo transformation into a proliferative phenotype.[27–29] Some evidence supports the contention that directly stretching these cells, as occurs with dilating force from an angioplasty balloon, is sufficient to produce the transformation. Possibly, conformational changes in myocyte membranes and distortion of surrounding matrix structures are the mechanisms responsible for initiating cellular activation. Other evidence suggests a role for inflammatory cells, such as neutrophils, lymphocytes, and macrophages. However, Schwartz et al.[18] have shown in porcine coronary arteries that even severe stretching of the media does not produce significant smooth muscle cell neointimal proliferation in this model unless the IEL is ruptured also. It is important to note that in human postmortem and atherectomy specimens most neointimal proliferation appears to occur in the plaque-free zone of the arterial wall, where the greatest tensile stress has been applied to apparently normal myocytes. Myocyte stretch injury is therefore probably involved to some extent in the transformation process.

Platelet Aggregation and Thrombus Formation

The earliest and most consistently reproducible finding after angioplasty, in experimental animal models as well as in human clinical studies, is platelet deposition at the angioplasty site. Endothelial denudation is virtually always accompanied by platelet deposition along the denuded area, even if only with a single layer of platelets. Occlusive thrombus does not always form, although it sometimes does; and intense efforts to prevent platelet deposition have not worked. With the endothelium missing, collagen, elastin, and other subendothelial microfibril elements bind to specific glycoprotein re-

ceptors on platelet surfaces, and this reaction apparently is so fundamental that even induction of severe thrombocytopenia does not completely prevent it.[30] Platelets in the adherent layer become activated by surface receptor binding, undergo conformational changes, and release various mediators from their storage granules. Some of these released substances, such as thromboxane A_2, thrombin, serotonin, and adenosine diphosphate, stimulate further aggregation of platelets onto the initial layer. Fibrinogen is converted to fibrin by thrombin, and fibrin strands are deposited onto the platelet mass and begin to cross-link and polymerize. A thrombus of variable size may form. A large, fully formed thrombus may completely occlude the vessel at the angioplasty site. This event, clinically termed *abrupt closure*, occurs in 5% to 25% of patients, depending on several clinical and anatomic factors.

The possible association between nonocclusive thrombus formation and later restenosis is not clear. Halon et al.[31] found that restenosis occurred in 67% of patients with intracoronary thrombus visible at the site of angioplasty, compared with restenosis in 19% of patients without intracoronary thrombus. However, Hirshfeld et al.[32] noted restenosis in 40% of patients without thrombus at the angioplasty site but in only 27% of patients with thrombus. Because platelet deposition is universal, there must be some thrombus, albeit subclinical in many cases, at all angioplasty sites. Angioscopic studies have supported this contention.[18,20] Restenosis is not universal, however, which implies that there must be other factors involved in addition to platelets and thrombus. It is also possible that platelet-fibrin thrombi (even microthrombi) could form a "skeleton" on which smooth muscle cells and macrophages attach and begin the proliferative and secretory actions that eventually lead to neointimal hyperplasia.[9,14] Studies in our laboratories have provided additional evidence for the importance of platelets in the restenosis process.[33] By means of the Folts model of coronary stenosis with endothelial injury, cyclic coronary arterial flows were established in 24 canine coronary arteries. Thromboxane receptor antagonists, thromboxane synthetase inhibitors, serotonin receptor antagonists, or combinations of these agents were administered to eliminate cyclic flows by interfering with platelet functions. Animals with few or no cyclic flows (good response to treatment agents) were found at postmortem examination after 21 days to have extremely limited neointimal hyperplasia. Animals with strong and intense cyclic flows (poor response to treatment agents or no treatment) were found to have marked neointimal hyperplasia. Animals with intermediate and weak cyclic flows were found to have intermediate degrees of hyperplasia. A proportionality thus existed between the intensity of cyclic flows from platelet accumulation and subsequent neointimal proliferative response in this animal model.

Release of Growth Factors

Storage granules of platelets adherent to angioplasty sites release a number of mediators that promote healing and repair of the arterial wall, ultimately stimulating growth of new tissues. Several of these growth factors are listed in Table 1.[34–39] The most well characterized of these, platelet-derived growth factor (PDGF), stimulates chemotaxis of neutrophils and macrophages, as well as the transformation of smooth muscle cells to a noncontractile or "synthetic" phenotype.[40,41] Platelets also release serotonin, thrombospondin, and transforming growth factor 1, all of which are mitogenic for smooth muscle cells.[14] Neutrophils and macrophages attracted into the injured region also release a variety of these growth factors as components of the inflammatory response. Furthermore, injured and dying smooth muscle cells may release growth factors (especially basic fibroblast growth factor) that could serve to stimulate the remaining live myocytes in the region.[14,36,42] All of these growth factors and cytokines probably act in concert in as yet poorly defined ways to produce the neointimal hyperplasia of restenosis. Their activities are probably maximal early on, from day 1 to days 3 or 4 after injury, and then subside. Once initiated, the hyperplastic response continues until it is completed, which appears to require about 4 to 20 weeks.

Inflammation

Leukocyte infiltrates, mostly monocytes and macrophages but also neutrophils, are another important component of the arterial wall response to angioplasty injury. These cells probably are recruited directly from the bloodstream by release of chemotactic factors from platelets and injured smooth muscle cells.[8,9,14] It is intriguing and relevant to note that injury of the medial and adventitial layers of arterial wall alone, without endothelial denudation, appears to be sufficient to produce a localized inflammatory response that induces smooth muscle cell proliferation,[43–45] attesting to the universality of this process. This inflammation probably occurs as a result of release

TABLE 1. Selected Growth Factors and Cytokines Involved in Arterial Repair and Restenosis After Coronary Angioplasty

Substance	Source	Reference
PDGF	Platelets, SMC	Ross et al.[34]
Serotonin	Platelets	Nemecek et al.[35]
Transforming growth factor	Platelets	Casscells[38]
Basic fibroblast growth factor	Macrophages, SMC	Casscells et al.[37]
Insulin-like growth factor	SMC	Cercek et al.[36]
Interleukins 1 and 6	Macrophages	Loppnow et al.[39]

SMC, Smooth muscle cells.

of chemotactic factors by stretching of myocytes or disruption of their surrounding proteoglycan matrix. Production and release of free radicals may also be involved. Macrophages, recruited as part of the inflammatory response, synthesize and secrete various growth factors for smooth muscle cells, including PDGF, basic fibroblast growth factor, transforming growth factor, interleukin-1, and heparin-binding growth factor.

Transformation of Smooth Muscle Cells

The single most fundamental element in the process of restenosis is the transformation of some smooth muscle cells in the tunica media from a contractile to a proliferative, secretory phenotype. Atherosclerotic plaques do appear to have at least some transformed smooth muscle cells already acting in place of surface endothelial cells by providing a partial nonthrombogenic lining. However, these probably are not the cells responsible for the hyperplastic neointimal response that characterizes restenosis. The majority of the smooth muscle cells involved in the restenosis process are probably recruited by the actions of inflammation and growth-factor release just described. Normally, the smooth muscle cells of the tunica media are rather quiescent. In the contractile phenotype, they have abundant myofibrils but virtually no subcellular machinery for synthesis and secretion (ribosomes, rough endoplasmic reticulum, Golgi bodies). These smooth muscle cells provide structural support for the vessel wall, as well as defining its resting tone by their responsiveness to vasoactive influences. Angioplasty injury—with the aforementioned sequence of events: endothelial denudation, platelet deposition, myocyte stretch, disruption of media, invasion of inflammatory cells, and release of growth factors and cytokines—stimulates a subpopulation of myocytes in the subjacent and nearby medial layers to undergo the transformation to a proliferative, secretory phenotype. These transformed myocytes are more similar to fibroblasts, and develop extensive subcellular organelles for proliferation and the secretion of extracellular matrix materials. In the contractile phenotype, before transformation, the proteoglycan matrix surrounding each myocyte probably helps maintain its quiescent status, possibly because of heparin or heparin-like molecules. Disruption of the proteoglycan matrix of the medial layers, along with endothelial denudation and breach of the IEL, sets the stage for removal of these growth-inhibitory heparinoids.[8,14] Along the denuded surface and within the crevices of the damaged medial layers, platelet degranulation releases endoglycosidases that destroy many of the heparinoids.[46,47] This loss of inhibitory control, together with the delivery of numerous growth factors and stimulative cytokines, promotes the transformation of the smooth muscle cells. Removal of the heparinoids may expose smooth muscle cell surface receptors capable of binding many of the growth

factors, and proteoglycan fragments in the matrix could bind some factors and increase their local concentrations. By 48 hours after angioplasty injury, smooth muscle cells in the tunica media can be found to be synthesizing DNA and developing secretory organelles, whereas in an uninjured arterial wall fewer than 0.1% of the smooth muscle cells synthesize DNA. Only a small proportion of the smooth muscle cells in the medial layer at the angioplasty site or in nearby layers, however, ultimately undergo the transformation. The vast majority of smooth muscle cells in the tunica media at the angioplasty site appear to remain in the quiescent, contractile phenotype.

Migration of Smooth Muscle Cells

After angioplasty injury, some of the transformed smooth muscle cells of the media begin to migrate to the intima. Whether these smooth muscle cells come only from injured media directly subjacent to the angioplasty site or may also be attracted to migrate along the surface from adjacent areas is not entirely clear, but there is evidence to support both views.[8,9,45] By 4 days after angioplasty, both light and electron microscopy have shown transformed smooth muscle cells moving into the intima (Fig. 4). Fibroblasts within the media almost certainly also make this trek. The directed migration of these cells, whether from subjacent layers or from other nearby sites, is made in response to chemotactic factors released in the intima. These cells do not migrate to the adventitial zone, even though this zone is stretched and distorted just as much as the inner layers. This strongly implicates the damaged endothelium or platelets and formed thrombus that accumulates along the intimal surface after injury. Probably the endoglycosidase released by platelets that helps destroy the heparin-containing proteoglycan matrix also helps with the deadherence of smooth muscle cells from the matrix and from neighboring cells, making migration possible. Some of the other substances released by platelets, especially PDGF and transforming growth factor, are chemotactic for smooth muscle cells, at least in culture and probably also in vivo.[36,41,48] Activated smooth muscle cells themselves secrete PDGF-like molecules, which can act on surrounding smooth muscle cells and thus help to sustain migration. The other cells attracted into the injured intimal zone as part of "inflammation," neutrophils and macrophages, also secrete chemotactic agents. Breaks in the IEL caused by the injury may open routes for the migrating cells. It is also possible that several products released by platelets, macrophages, and transformed smooth muscle cells—such as fibrinolytic agents and hyaluronidase—help both to erode the IEL and to degrade other adhesive components of the arterial wall.

Transformed smooth muscle cells migrate for about 2 to 3 weeks after angioplasty injury. There is some evidence that slowly degenerating thrombus at the angioplasty site is a continuing (albeit slowly

declining) source of growth factors and chemotactic agents during this period. According to this hypothesis, the thrombus 'skeleton' is gradually colonized by macrophages and lymphocytes as well as some transformed smooth muscle cells, all recruited by the actions of growth factors and chemotactic agents.[9] The macrophages secrete specific fibrinolytic enzymes that aid in the gradual resorption of the thrombus.[49] It is also known, however, that transformed smooth muscle cells secrete plasminogen activators and could thus assist with the resorption of thrombus.[50] Either way, the thrombus gradually disappears, to be replaced eventually by an amorphous mixture of rather nondescript cells along with copious amounts of extracellular matrix material. Although the migration may cease after 2 to 3 weeks, production of extracellular matrix can continue for much longer, possibly as long as 20 to 25 weeks.

Proliferation of Smooth Muscle Cells

Some of the smooth muscle cells activated by angioplasty injury and the actions of growth factors and cytokines will enter the cell cycle, undergo mitosis, and proliferate. It is extremely likely that the activities of migration and proliferation are separate, in that some migrating smooth muscle cells do not proliferate and some proliferating smooth muscle cells do not migrate.[14] The same growth factors and cytokines that stimulate transformation of smooth muscle cells into the proliferative, secretory phenotype are the mitogenic factors responsible for proliferation. Only about one half of the smooth muscle cells activated and transformed actually undergo proliferation, however, perhaps indicating substantial growth inhibition present in these tissues. As mentioned, heparin-like compounds in the proteoglycan matrix surrounding smooth muscle cells probably help to maintain quiescence. Loss or disruption of these compounds by physical forces and the action of endoglycosidases, combined with the delivery of various growth factors, removes some growth inhibition, at least temporarily. The smooth muscle cells entering the cell cycle appear to undergo an average of about three divisions each before growth control is restored. Cells located along the deendothelialized subintimal surface appear to have a much greater proliferative potential than do cells from deeper layers.[51] This may reflect greater exposure to stimulative factors for subintimal cells, greater inhibitory control of deeper cells, or both.

The proliferative activities of transformed smooth muscle cells reach a peak between 2 to 4 weeks after angioplasty, then subside and appear to be diminishing by about 8 to 12 weeks. Evidence of proliferation may persist until 26 weeks or even longer.[8] Gradually the smooth muscle cells lose their synthetic phenotype appearance and assume the appearance of quiescent mesenchymal cells, although they do not resume the appearance of purely contractile type smooth muscle cells that they had before transformation.

Production of Extracellular Matrix

The second fundamental element of restenosis, after cellular transformation, is the production and secretion of extracellular proteoglycan matrix. Smooth muscle cells that have migrated into the injured intimal zone or colonized the degenerating thrombus begin to produce several proteoglycans. These include chondroitin sulfate, dermatan sulfate, and heparin sulfate. To some extent, locally active fibroblasts also produce these compounds. These secreted proteoglycans replace fibronectin as the major extracellular matrix material in this region.[8] Synthesis of both chondroitin sulfate and dermatan sulfate are regulated by transforming growth factor β. On the other hand, the third proteoglycan, heparin sulfate, which is a product of both endothelial cells and smooth muscle cells, does not appear to be regulated by transforming growth factor β. Interestingly, although heparin sulfate is a potent inhibitor of smooth muscle cell proliferation and normally helps maintain these cells in a quiescent state, it also appears to stimulate proteoglycan synthesis by activated smooth muscle cells. With time, weeks or months after angioplasty injury, much of this newly synthesized proteoglycan matrix is gradually replaced by collagen and elastin.[52] Synthesis of the new proteoglycan gradually ceases, and the histologic picture comes to resemble a scar such as might develop in any wound. Histologic specimens from experimental animal models, from human postmortem samples taken from patients dying shortly after angioplasty, and from atherectomy specimens of restenosis lesions have all demonstrated sparse mesenchymal cells wrapped in abundant connective tissue matrix (Fig. 5). According to calculations by Schwartz et al.,[9] cellular components constitute only about 11% of neointimal volume; extracellular matrix constitutes the remainder. Even though smooth muscle cell migration and proliferation are important features of the restenosis process and no doubt set the stage for matrix production, it thus is the extracellular matrix material that forms the "bulk" of restenosis, both literally and figuratively. It is possible that major strides could be made in reducing clinical restenosis if the synthesis of this extracellular matrix material by activated smooth muscle cells could be safely slowed or stopped.

Regrowth of Endothelium

The hyperplastic neointimal growth response to angioplasty injury gradually ceases. Probably one extremely important factor in this termination phase is the regrowth of endothelial cells over the luminal surface of the vessel. In cell culture studies, endothelial cells will pro-

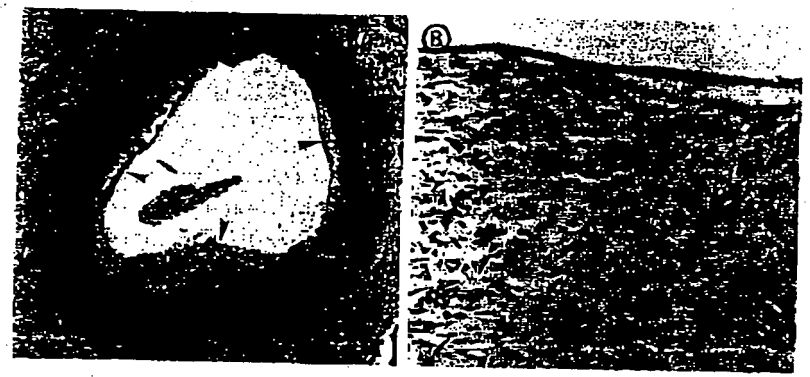


FIG. 5. Photomicrographs of cross-sections of left anterior descending coronary artery from a dog. The dog was killed 3 weeks after balloon angioplasty performed at the site of an externally placed coronary constrictor. **A,** The canine coronary artery at the site of angioplasty exhibits multiple areas of neointimal thickening (arrowheads). (Original magnification ×16; hematoxylin-eosin stain). **B,** At higher magnification, the neointimal proliferative lesion is seen to be composed of altered and elongated smooth muscle cells, along with abundant connective tissue matrix. The lumen is lined by plump endothelial cells. This experimentally produced lesion has histopathologic features that resemble those seen in human beings at coronary angioplasty sites. (Original magnification ×92; hematoxylin-eosin stain. Courtesy of Dr. L. M. Buja, University of Texas Health Science Center at Houston.)

liferate to form a confluent monolayer, after which their proliferation stops.[53] In experimental animal models, when gentle endothelial denudation of the luminal surface of an artery is performed, reendothelialization occurs within about 7 days and there is little or no neointimal hyperplasia.[14, 16] Conversely, when deeper injury occurs such that the IEL is broken and smooth muscle cells of the media are exposed to blood, or a sufficiently large enough area is denuded, endothelial cell regrowth is delayed and may be incomplete. Some areas may then remain uncovered by endothelial cells and a thick neointima can develop. In fact, endothelial cell regrowth can be arrested by substances released by activated smooth muscle cells.[17, 54, 55] Thus on the one hand the proliferating endothelial cells can inhibit smooth muscle cell proliferation, whereas on the other hand the proliferating smooth muscle cells can inhibit endothelial cells. It is interesting that proliferating endothelial cells secrete heparin sulfate, which helps maintain quiescence and inhibit proliferation of smooth muscle cells but also stimulates proteoglycan synthesis by transformed smooth muscle cells. The period following angioplasty in one sense resembles a race between endothelial cells and smooth muscle cells, with both groups attempting to repopulate the neointimal zone of injury. Clinically, in patients dying soon after coronary angioplasty, incomplete reendothelialization of the angioplasty site was noted in

patients dying less than 30 days later, whereas complete reendothelialization was noted to have occurred in patients dying more than 30 days later.[43] One possible explanation for the events that follow angioplasty injury is that circumscribed endothelial denudation without deep medial injury permits proliferating endothelial cells to cover the region rapidly. Conversely, widely extensive endothelial denudation, deep medial injury, or both, permit migrating smooth muscle cells to arrive and establish themselves in the neointima before proliferating endothelial cells can arrive. However, there apparently are limits to continued hyperplastic neointima formation. Even in areas chronically denuded of endothelium, neointimal thickness peaks at about 8 weeks after injury and then remains stable.[17, 51] This may have clinical parallels, inasmuch as restenosis almost never progresses to occlude the artery completely, regardless of the length of the follow-up interval, suggesting that there are indeed limits to the proliferative process. Whether this eventual stabilization represents some equilibrium between activated endothelial cells and continuously proliferating smooth muscle cells or a loss of replicative capacity in both cell types is not clear. An equilibrium of some sort appears to be the mechanism, though, because the substrate of smooth muscle cells in areas chronically denuded of endothelium has a higher proliferation rate than that seen in truly quiescent areas (6% per day vs 0.1% per day). If complete reendothelialization of the luminal surface does occur, contact by smooth muscle cells with blood and blood-borne mitogens ceases. Thrombus dissolves and thrombus-derived growth factors disappear. Proteoglycan matrix accumulates and gradually plateaus at some stable amount. With or without complete reendothelialization, however, the restenosis process appears to terminate slowly after about 20 to 25 weeks.

EPIDEMIOLOGY OF CLINICAL RESTENOSIS

More than a decade of clinical research work has been spent on identifying and understanding factors associated with restenosis and on possibly reducing the incidence of restenosis by employing various therapies. A recent literature review[58] evaluated 212 published reports, the equivalent of the appearance of almost two publications per month for 10 years. Despite the time and energy (not to mention money) spent on the clinical restenosis problem, numerous pitfalls and caveats still exist. Some of these are described here. The problem of restenosis is as large as ever. All is not gloom and doom, however, for a number of important advances in both experimental observation and clinical thinking have occurred.

DEFINING CLINICAL RESTENOSIS

Because coronary arterial lesions are identified and treated with the use of angiography, one might hope that an angiographic definition of restenosis exists. In fact, there are at least six different definitions of restenosis that have been used commonly in various studies. These are shown in Table 2. Serruys et al.[57] have counted 12 different definitions of restenosis. As leaders in this field, they have also presented compelling data to suggest that the change in minimum lumen diameter is the proper variable to consider for restenosis. The existence of so many definitions of restenosis has led to confusion. Restenosis by one set of criteria is not necessarily equivalent to restenosis by another set of criteria. Fig. 6 illustrates some of the possible classifications for three representative lesions according to five of the common set of restenosis criteria. The lack of a uniform angiographic definition of restenosis is a major limitation to studies of the problem and presents quite a challenge when attempting to apply basic biologic principles, as discussed in the previous sections, to the clinical observations. The fact that investigators use a single definition for their group comparisons—and as a result their studies are at least internally consistent—is helpful. Problems arise when attempting to compare and contrast different studies. It is to be hoped that a uniform angiographic definition of restenosis will be devised, validated, and accepted in the not-too-distant future.

DETECTING CLINICAL RESTENOSIS

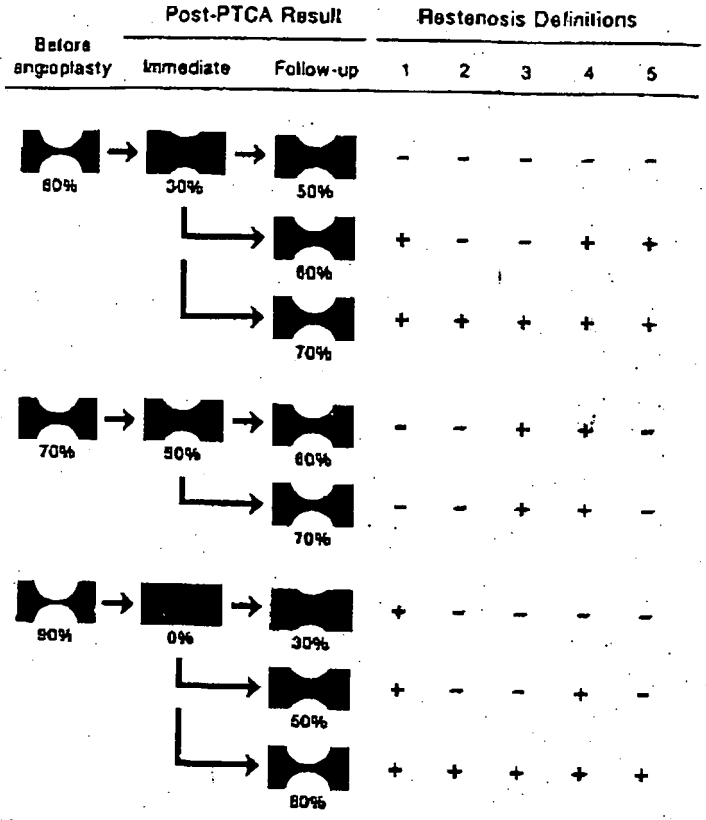
Symptoms

Many patients with coronary disease have symptoms of either stable or unstable angina pectoris. They gain relief from their symptoms

TABLE 2. Several Commonly Used Arteriographic Definitions of Restenosis After Coronary Angioplasty

1. An increase of ≥30% from stenosis immediately after angioplasty to follow-up stenosis (NHLBI-1)
2. An initial diameter stenosis <50% after angioplasty, increasing to ≥70% at follow-up angiography (NHLBI-2)
3. An increase in diameter stenosis at follow-up angiography to within 10% of the preangioplasty value (NHLBI-3)
4. A loss of ≥50% of the initial diameter stenosis gain achieved by angioplasty, from immediately after angioplasty to follow-up angiography (NHLBI-4)
5. A postangioplasty diameter stenosis <50% increasing to >50% at follow-up angiography
6. A decrease in the minimal luminal diameter at the lesion of >0.72 mm from immediately after angioplasty to follow-up angiography

NHLBI, National Heart, Lung, and Blood Institute Percutaneous Transluminal Coronary Angioplasty Registry.



Definition 1: An increase in diameter stenosis of at least 30% from immediately post-PTCA at the time of follow-up.
Definition 2: An immediate post-PTCA diameter stenosis of less than 50% increasing to greater than or equal to 70% at follow-up.
Definition 3: An increase in stenosis severity to within 90% or less of the diameter stenosis before PTCA at the time of follow-up.
Definition 4: A loss of at least 50% of the gain in luminal diameter achieved immediately post-PTCA.
Definition 5: An increase in diameter stenosis from less than 50% immediately after PTCA to greater than 50% at follow-up.

FIG. 6. Illustration of possible arteriographic findings with angioplasty and at follow-up. Three representative lesions are shown. At follow-up, restenosis may or may not be judged to have occurred, depending on the angiographic characteristics of the lesion and the definition of restenosis used. The five definitions of restenosis shown are the first five definitions listed in Table 2. *PTCA*, Percutaneous transluminal coronary angioplasty; −, restenosis absent; +, restenosis present. (Adapted from Califf RM, Ohman EM, Frid DJ, et al. Restenosis: the clinical issues. In: Topol EJ, ed. Textbook of interventional cardiology. Philadelphia: WB Saunders, 1990:363–94. Used with permission.)

with successful angioplasty of the involved artery or arteries. Recurrence of anginal symptoms is thus worrisome, for it suggests recurrence of a coronary lesion. This may be especially true in patients with multivessel disease or in patients with nonsignificant disease in several vessels, where many combinations of recurrences or disease progression can occur. However, symptom status has not been found to be a useful clinical indicator overall for restenosis (Table 3).[58–65] In general, symptoms recur more frequently than do significant coronary lesions. The proportion of patients with symptoms who have been found at follow-up angiography actually to have restenosis (the positive predictive value of symptoms) has ranged from 48% to 92%, with an average of 67%, or two thirds. The proportion of patients without symptoms found to be free of restenosis (the negative predictive value of symptoms) has ranged from 70% to 98%, with an average of 85%. This also means that approximately one third of patients with symptoms will be free from restenosis, whereas approximately 15% of patients without symptoms actually will have restenosis ("silent restenosis"). These figures for predictive values are much lower than is clinically desirable. They may be explained, in part, by the existence of numerous mechanisms for chest pain syndromes. From a practical standpoint, a clinician cannot rely on symptoms alone but must couple them (or their absence) with other data.

Noninvasive Testing

Reliable and accurate noninvasive tests for restenosis are obviously desirable. Unfortunately, currently available noninvasive tests are far from ideal. Exercise testing, both alone (Table 4)[64, 66–74] and coupled with thallium 201 scintigraphy (Table 5),[58–72, 75–78] has been found to be limited by predictive values that are lower than one would hope

TABLE 3. Value of Symptoms for Detecting Restenosis After Coronary Angioplasty

Reference	Number of patients	Number with angiographic follow-up	Restenosis rate (%)	Symptoms reported (%)	Value of symptoms	
					PPV (%)	NPV (%)
Gruentzig et al.[58]	133	124	31	39	92	98
Simonton et al.[59]	228	206	35	93	48	75
Levine et al.[60]	100	92	40	46	76	96
Mabin et al.[61]	153	84	32	28	71	86
Zaidi et al.[62]	184	184	49	95	66	70
Holmes et al.[63]	524	524	34	48	58	86
Bengtson et al.[64]	263	263	38	41	60	85
Range			31–49	28–95	48–92	70–98
Average			37	56	67	85

PPV, Positive predictive value; *NPV*, negative predictive value.
Adapted with permission from Califf RM, Ohman EM, Frid DJ, et al. Restenosis: the clinical issues. In: Topol EJ, ed. Textbook of interventional cardiology. Philadelphia: WB Saunders, 1990:363–94.

TABLE 4. Value of Exercise Testing for Detecting Restenosis After Coronary Angioplasty

Reference	Number of patients	Number with angiographic follow-up	Restenosis rate (%)	Value of test	
				PPV (%)	NPV (%)
Very early (<1 week)					
El-Tamimi et al.[66]	31	31	45	79	82
Early (1 week–2 months)					
O'Keefe et al.[67]	48	48	13	29	73
Wijns et al.[68]	120	89	35	50	65
Wijns et al.[69]	87	77	40	60	52
Scholl et al.[70]	36	30	12	40	27
Later (2–12 months)					
Ernst et al.[71]	25	25	4	50	95
Rosing et al.[72]	100	100	34	47	76
Bengtson et al.[64]	209	200	25	61	84
Honan et al.[73]	164	144	40	57	64
Pirelli et al.[74]	75	75	19	29	90
Range			4–45	29–79	27–95
Average			27	50	71

PPV, Positive predictive value; *NPV*, negative predictive value.
Adapted with permission from Califf RM, Ohman EM, Frid DJ, et al. Restenosis: the clinical issues. In: Topol EJ, ed. Textbook of interventional cardiology. Philadelphia: WB Saunders, 1990:363–94.

for widely applicable tests. The negative predictive values (proportion of patients with negative test results who do not have restenosis) are better than the positive predictive values, both for plain exercise testing and for exercise thallium 201 scintigraphy, affirming the usefulness (albeit moderate) of the negative result of a noninvasive study. A number of confounding factors probably influence this lack of utility. These include the use of angioplasty in patients with multivessel disease or previous infarction, in whom positive or negative test results may be more difficult to interpret. Also, problems with inadequate levels of stress, the coexistence of conditions such as left ventricular hypertrophy or conduction defects, and the use of medications such as β-adrenergic blocking agents or calcium-channel antagonists, can all influence test results and cloud interpretation. Although some investigators have suggested that early exercise testing (less than 1 month and sometimes less than 1 week) after angioplasty can help predict early restenosis, this has not been universally reported. Positive results of exercise tests early after angioplasty, including exercise thallium 201 scintigrams, have been associated with small-vessel vasoconstriction, altered vasomotor tone, and "delayed resolution" of the previous abnormality.[79–81] It is probably more reasonable to wait at least 2 to 4 months after angioplasty before attempting to identify restenosis through noninvasive testing, if the clinical and coronary anatomic picture is otherwise acceptable. Otherwise, if results of early noninvasive testing are positive and repeat angiography is performed

TABLE 5. Value of Thallium 201 Scintigraphy for Detecting Restenosis After Coronary Angioplasty

Reference	Number of patients	Number with angiographic follow-up	Restenosis rate (%)	Value of test	
				PPV (%)	NPV (%)
Very early (<1 week)					
Hardoff et al.[73]	90	71	32	53	86
Jain et al.[74]	40	22	14	79	88
Early (1 week–2 months)					
Lam et al.[77]	43	43	9	89	96
Wijns et al.[68]	120	89	35	74	83
Wijns et al.[69]	87	77	40	82	72
Scholl et al.[70]	38	30	12	56	42
Later (2–12 months)					
Ernst et al.[71]	25	25	4	50	100
Rosing et al.[72]	58	58	21	37	83
Hecht et al.[75]	116	116	60	85	88
Range			4–60	37–89	42–100
Average			25	67	82

PPV, Positive predictive value; *NPV*, negative predictive value.
Adapted with permission from Califf RM, Ohman EM, Frid DJ, et al. Restenosis: the clinical issues. In: Topol EJ, ed. Textbook of interventional cardiology. Philadelphia: WB Saunders, 1990:363–94.

with negative results, the whole cycle would probably have to be repeated again after about 6 months because the biologic processes of restenosis can take that long to become manifest. From a practical standpoint, in a patient without symptoms, a negative result from an exercise thallium 201 scintigram with an acceptable level of stress more than 4 months after angioplasty has better than 90% predictive value for absence of restenosis. Although not applicable to all patients undergoing coronary angioplasty, the strategy of later (4 to 6 months) noninvasive testing with scintigraphy can be applied to many. Angiography could be reserved for those patients who are not able to undergo scintigraphy, those whose lesions are complicated or known not to produce remarkable imaging results, and those whose noninvasive test results are indeterminate, equivocal, or definitely positive.

Angiography

Repeated coronary arteriography at some point after a successful angioplasty, comparing the follow-up images with the immediate result, has been the reference standard for determining restenosis. As we have seen, there is no single accepted definition of restenosis based upon the comparison of these arteriographic images, yet virtually all investigators agree on the need for angiography to make the decision. The timing of follow-up angiography is important. It must not be done so early as to miss developing restenosis, yet not so late that important recurrent coronary disease is left unappreciated. By employing serial angiography, both Nobuyoshi et al.[82] and Serruys et al.[83] demonstrated that the incidence of restenosis increases each month after successful angioplasty, until it plateaus after about 4 months (Fig. 7). The incidences shown in Fig. 7 are based on the definition of restenosis as the loss of more than one half of the initial gain in luminal diameter stenosis achieved by the angioplasty procedure. Other definitions could have been applied, and in fact Serruys et al.[83] showed with their own data that the overall restenosis rate at 4 months varied between 2.8% and 30.2% depending on which of the six common definitions was used.

CLINICAL CORRELATES OF RESTENOSIS

Many clinical studies have been performed to identify correlates of restenosis (or, taking the opposite view, lack of restenosis). Traditionally, they have been divided into three different categories of variables: clinical, patient-related factors; coronary lesion–related factors; and angioplasty procedure–related factors. For excellent reviews of the clinical correlates of restenosis, as well as critiques of the many studies from which they were derived, see Bobbio et al.,[56] Califf et al.,[55] Fanelli and Aronoff,[84] and Hermans et al.[85]

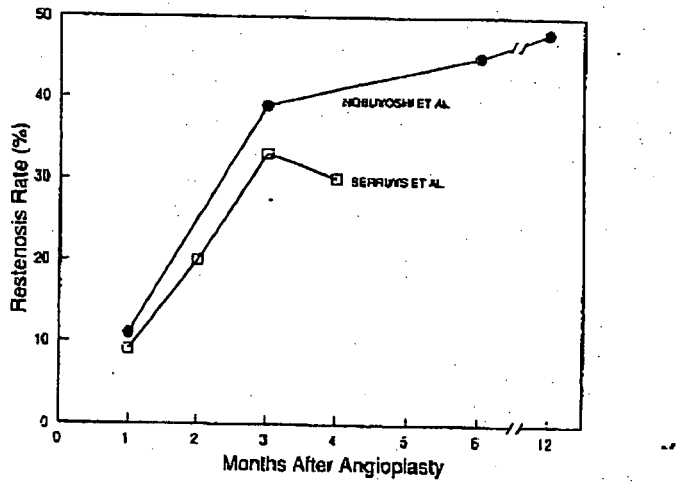


FIG. 7. Incidence of restenosis by serial angiography after coronary angioplasty. The angiographic definitions of restenosis are the same (loss of >50% of the immediate gain achieved by angioplasty). (Data reprinted with permission from the American College of Cardiology, Nobuyoshi M, Kimura T, Nosaka H, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: serial angiographic follow-up of 229 patients. J Am Coll Cardiol 1988;12:616–23. Also from: Serruys PW, Luijten HE, Beatt KJ, et al. Incidence of restenosis after successful coronary angioplasty: a time-related phenomenon. Circulation 1988;77:361–71. Data used with permission.)

Patient-Related Factors

A number of specific patient-related variables appear to be associated with increased risks of restenosis. These include male gender, unstable angina, diabetes, and continued smoking after angioplasty. Several other factors known or suspected to be involved in development or progression of coronary artery disease have not been shown to be associated with restenosis or appear to have much weaker associations. These others include family history of coronary disease, hypertension, and abnormal lipid profiles.

Lesion-Related Factors

Lesion-related factors include multilesional and multivessel angioplasty procedures, higher postangioplasty residual stenosis, ostial lesions, proximal vessel lesions, location in the left anterior descending artery, location in a saphenous vein graft, longer lesions, and total occlusions.

Procedure-Related Factors

Procedure-related factors include higher postangioplasty residual stenosis, along with suboptimal balloon-to-artery diameter ratios (balloon less than approximately 90% or greater than approximately 115% of the adjacent "normal" artery). Many other procedure-related factors, such as number of balloon inflations, duration of balloon inflations, and balloon pressures, have not been found consistently to be related to higher risk for restenosis and may in fact be related more to inability to achieve a good result, which itself may be only indirectly related to restenosis. One other procedure-related factor that has received a great deal of attention has been tear or dissection of the treated coronary artery. Although dissection can be angiographically dramatic at times and may increase the risk of abrupt closure, by itself it does not appear to increase the risk of subsequent restenosis.[86–88] The important element may be that a successful initial result was achieved, with low residual stenosis values[88] or a low transstenotic pressure gradient and good flow,[86] and not the presence or absence of dissection per se.

LIMITATIONS OF CLINICAL RESTENOSIS STUDIES

Clinical restenosis studies have suffered to various degrees from methodologic problems. Many studies are quite small, following fewer than 100 patients after successful coronary angioplasty. Many have enrolled or followed heterogeneous patient groups. The timing and method of follow-up for restenosis studies have also been quite varied. Some follow-up intervals have been as short as 3 months, whereas others have been 6 or even 12 months. Biologic issues discussed in the previous sections, as well as the clinical work of Nobuyoshi et al.[82] and Serruys et al.,[83] seem to indicate that a clinical follow-up interval of 6 to 12 months is probably most appropriate. To be complete, a restenosis study should have 100% angiographic follow-up. This is at least extremely difficult—and may indeed be impossible—for social, technical, and financial reasons. In a comprehensive review of clinical restenosis studies,[56] only four out of 31 carefully screened reports had 100% angiographic follow-up, although six others had greater than 90% angiographic follow-up. The largest of the complete studies had only 82 patients, and the smallest had 19 patients. Califf et al.[89] demonstrated that observed restenosis rates in one population could vary from a value of 33% to a value of 44%, depending only on the mix of patients included when follow-up angiography is less than complete. Indeed, many restenosis studies have relied on retrospective reviews of patients who happened to undergo coronary arteriography at some point after a successful angioplasty procedure. Often, this angiogram may have been initiated because the patient had return of symptoms or an exercise test was performed and the results were either indicative of ischemia or indeterminate. Selection bias in choosing patients for follow-up angiography can therefore cloud the results of restenosis studies.

The final and major problem with clinical correlative restenosis studies is the fact that they can be misleading if applied too readily to patient care. All patients undergoing coronary angioplasty must have some follow-up evaluation. No constellation of variables has yet been found that indicates complete freedom from restenosis. Conversely, no group of variables has been identified that will make restenosis absolutely certain. The majority of patients have mixtures of variables that are both favorable and unfavorable toward restenosis. In one sense, then, restenosis variable definition is irrelevant because it will not help in individual patient care. Identifying these variables may be useful from a research standpoint, especially because it can help keep therapeutic trials balanced. From a practical standpoint, however, it is only the anatomic location of the lesions that is paramount: patients who have important coronary disease are always at risk for recurrence or progression of their important disease.

THERAPIES TO PREVENT RESTENOSIS

A number of studies have been performed during the past years to try to prevent restenosis. The major categories are shown in Table 6. Some of these studies suffer from many of the same methodologic problems that have plagued clinical correlation studies. This includes small sample sizes, incomplete follow-up, and lack of a uniform definition of restenosis. Still, these are the only data available. The trials are discussed here by class of therapeutic compound studied.

TABLE 6. Categories of Therapeutic Agents Evaluated for Prevention of Restenosis After Coronary Angioplasty

Antiplatelet agents
Aspirin
Dipyridamole
Ticlopidine
Combinations
Anticoagulants
Heparin
Warfarin (coumadin)
Prostanoids
Fish oils
Calcium-channel antagonists
β-Adrenergic receptor antagonists
Steroids
Thromboxane inhibitors
Serotonin inhibitors
PDGF antagonists
Angiotensin-converting enzyme inhibitors
Lipid-lowering agents
Antiinflammatory antioxidants
Thrombolytic agents
Antimitotic agents

Antiplatelet Agents

Although antiplatelet agents such as aspirin and dipyridamole have been shown to reduce the incidence of abrupt vessel closure with coronary angioplasty,[90,91] they have not been shown to reduce the incidence of subsequent restenosis. The main agents investigated have been aspirin (at various dose levels), ticlopidine, and dipyridamole, sometimes alone and sometimes in combination. Table 7 lists several representative studies.[92–102] From what is known about the involvement of platelets and thrombus with angioplasty injury to the vessel wall, it is not surprising that so many studies of antiplatelet agents would be undertaken. The results in general have been disappointing, although a few studies have indeed shown a beneficial effect on restenosis rates. The study by Kitazume et al.[102] in particular showed a substantial lowering of restenosis rates when aspirin and dipyridamole were combined with the calcium-channel antagonist nicorandil.

Because most of the antiplatelet activity from the agents tested occurs at the level of aggregation and platelet adhesion to injured endothelium is not reduced by these agents, platelets are not prevented from attaching to the arterial wall and releasing the contents of stored granules. Further aggregation of platelets onto the initial adherent layer may be reduced by antiplatelet agents, however, so that it is possible that the total platelet burden at the angioplasty site could

TABLE 7. Studies of the Effects of Antiplatelet Agents on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Control		Treatment			Conclusion/ comment
			Agent	Restenosis rate (%)	Agent		Restenosis rate (%)	
White et al.[92]	236	176	Placebo	25	1. Ticl	2. ASA/Dip	1.39 2.33	Not effective
Ellis et al.[93]	469	258	ASA	41	ASA + Hep		41	Not effective
Schanzenbacher et al.[94]	79	79	Low-dose ASA	18	High-dose ASA		21	Not effective
Mufson et al.[95]	495	165	Low-dose ASA	47	High-dose ASA		51	Not effective
Schwartz et al.[96]	376	249	Placebo	39	ASA/Dip		38	Not effective
Dyckmans et al.[97]	203	86	Low-dose ASA	31	High-dose ASA		21	Trend
Finci et al.[98]	40	31	Placebo	14	ASA		33	Not effective
Schwartz et al.[99]	243	243	Placebo	32	ASA/Dip		31	Not effective
Taylor et al.[100]	216	212	Placebo	38	ASA		25	Effective
Bertrand et al.[101]	200	244	Placebo	41	Ticl		50	Not effective
Kitazume et al.[102]	309	287	ASA	38	1. ASA + Ticl Ticl + Nicor	2. ASA +	1.27 2.16	Effective combination

ASA, Aspirin; *Dip*, dipyridamole; *Hep*, heparin; *Nicor*, nicorandil; *Ticl*, ticlopidine.

be lessened by antiplatelet therapy. It is tantalizing in this regard that two studies[99,100] have shown that the amount of neointimal proliferation may have been less with aspirin than with placebo, even though the overall "restenosis rates" were the same. If so, this would be consistent with data from experimental animal models that suggest the amount of neointimal hyperplasia after angioplasty correlates with the amount of platelet deposition. It also holds out the possibility that perhaps more specifically focused forms of antiplatelet therapy may be successful (at least partially) in helping to reduce restenosis rates.

PDGF Antagonist

Triazolopyrimidine, or trapidil, is related pharmacologically to dipyridamole. It is also an inhibitor of PDGF, although the specific mechanisms of this action are not known. As pointed out earlier, it is likely that PDGF is one of the most important growth-stimulating factors released from storage granules of platelets attaching to the arterial wall at angioplasty injury sites. In an experimental animal model, trapidil-treated animals had less hyperplastic neointimal narrowing after angioplasty than did untreated animals.[103] Building on this, three clinical studies of trapidil use after angioplasty have been reported (Table 8),[104–106] two of them undertaken in Japan, where trapidil is used for treating patients with angina. All three studies showed lower restenosis rates in trapidil-treated patients than in conventionally treated patients. These encouraging clinical results should prompt other investigators to attempt repetition. They are also likely to stimulate research studies with inhibitors of other growth factors thought to be involved in the restenosis process.

Anticoagulants

Although platelets are the elements initiating thrombus formation after intravascular injury, the enzyme thrombin is required to convert fibrinogen to fibrin for deposition of fibrin strands onto the platelet plug and for subsequent maturation of the thrombus. Thrombin inhibition therefore offers another route through which to influence the accumulation of platelets and thrombus on the vessel wall. The

TABLE 8. Studies of the Effects of Trapidil, a PDGF Antagonist, on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Conclusion/comment
			Control	Treated	
Maresta et al.[104]	251	115	37	29	Trend
Nishikawa et al.[105]	160	137	38	20	Trend
Okamoto et al.[106]	97	72	42	19	Effective

two commonly available inhibitors of thrombin, coumadin and heparin, have been investigated for possible prevention of restenosis after angioplasty. Heparin, a glycosaminoglycan, also has been shown to have growth-inhibitory activities for smooth muscle cells (as discussed earlier) in addition to its antithrombin activity. Heparin therefore offers the theoretic advantage of inhibiting restenosis by two mechanisms. Unfortunately, neither coumadin nor heparin has been shown to reduce restenosis after coronary angioplasty (Table 9).[107–110] Although the study by Hirshfeld et al.[110] did show a graded decline in restenosis rates with increasing duration of heparin infusion after angioplasty, this appears to have been a retrospective subgroup analysis. Heparin is not a single molecule, but instead is a mixture of glycosaminoglycans of various weights. It can be fractionated, and there are low–molecular weight fractions of heparin that have antiproliferative properties but little or no anticoagulant activity. In some animal models, these low–molecular weight fractions were shown to inhibit the hyperplastic neointimal response to angioplasty.[111] Preliminary reports of a clinical trial in human beings have been discouraging.[112] Other thrombin inhibitors, such as hirudin and hirulog, as well as various other fractions of heparin, have yet to be tested in clinical trials.

Fish Oils (ω Fatty Acids)

Greenland Eskimos, and certain other populations that consume large amounts of fish, have been found to have lower incidences of coronary heart disease and higher incidences of bleeding diatheses than populations consuming a more typical Western diet. This may be due in part to the differences in lipid composition of the diets and the effects that these lipids have on platelet functions and bleeding times. The major lipid prostanoid precursors in Eskimo diets are

TABLE 9. Studies of the Effects of Anticoagulants on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Conclusion/comment
			Control	Treated	
Thornton et al.[107]	248	248	27	36	Not effective
Urban et al.[108]	110	85	33	25	Not effective
Ellis et al.[109]	416	258	37	42	Not effective
Hirshfeld et al.[110]*	209	209	—		Effective, but retrospective subgroup analysis.
<2				60	
2–17				48	
17–30				44	
>30				40	

*Heparin infusion, duration in hours.

eicosapentaenoic acid and docosahexaenoic acid ("fish oils"), whereas the major prostanoid precursor in the typical Western diet is linoleic acid. The arachidonic acid pathway processes the fish oils from the membrane phospholipid pool and produces thromboxane A_3, which has much less procoagulant biologic activity than the usual thromboxane A_2. However, the prostaglandin I_3 that is also produced from the fish oils appears to have just as much antiplatelet activity as the usual prostaglandin I_2. Platelets and endothelial cells should therefore be less thrombogenic when these fish oils constitute the major fraction of the lipid pool. There is also some evidence that fish oils alter and reduce the production of PDGF by endothelial cells,[113] which in turn may help inhibit the hyperplastic neointimal response to injury. For these reasons, several clinical trials of dietary supplementation with fish oils to prevent restenosis have been conducted (Table 10).[114–121] The results overall are quite encouraging. Only two trials showed no benefit from fish oil supplementation, whereas six others did find benefit. Unfortunately, not all of the fish oil trials have had regular angiographic follow-up, with some relying instead on clinical outcomes and exercise tests. The most extensive of the fish oil studies, by Bairati et al.,[121] was a double-blind, randomized, placebo-controlled trial, that also used quantitative analysis of coronary arteriograms. In this study, the restenosis rates were compared according to several definitions of restenosis, and the beneficial effects of fish oil supplementation were still apparent. The major drawbacks to the use of fish oils are that treatment for several days (preferably a week) before the angioplasty procedure is necessary to get the lipid pool replaced, and fish oils often cause gastric distress, bloating, gaseousness, and a bad taste.

Prostanoids, Thromboxane, and Serotonin

As mentioned previously, the arachidonic acid pathway of platelets and endothelial cells produces prostanoid products that have proaggregatory and vasoconstrictive effects (thromboxane), as well as

TABLE 10. Studies of the Effects of Fish Oils on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Conclusion/comment
			Control	Fish oil	
Slack et al.[114]	162	0	33	16	Clinical follow-up only
Dehmer et al.[115]	82	82	36	16	Effective
Milner et al.[116]	194	145	36	19	Clinical restenosis rates
Grigg et al.[117]	108	101	31	29	Not effective
Reis et al.[118]	204	159	23	32	Clinical restenosis rates
Olsson et al.[119]	82	82	36	16	Effective
Nye et al.[120]	108	99	30	11	Effective
Bairati et al.[121]	205	119	48	31	Effective

those that have antiaggregatory and vasodilator effects (prostacyclin). In addition, serotonin, stored in platelet granules and released during platelet activation, also has proaggregatory and vasoconstrictor effects. Several clinical trials have investigated the use of agents that alter the balance of these effects to reduce restenosis (Table 11).[122–131] Encouraging preliminary reports on the beneficial effects of prostacyclin analogs[122,123] were not confirmed by a larger trial.[124] Yabe et al.[125] showed that thromboxane A_2 synthetase inhibition demonstrated a trend toward reduced rates of restenosis in a small study, whereas two large trials[127,128] that employed quantitative angiography did not confirm any effects for thromboxane receptor antagonism. However, Feldman et al.[129] did demonstrate a reduction in clinical events after angioplasty in patients treated with a thromboxane receptor antagonist. Serotonin receptor blockade with ketanserin, a drug initially used in Europe to treat hypertension and angina, was not found to reduce rates of restenosis after angioplasty.[130,131]

Inhibitors of Angiotensin-Converting Enzyme

A number of investigators studying the proliferative activities of smooth muscle cells in culture made the observation that angiotensin appeared to be a mitogen, or perhaps a comitogen.[132] These findings were rapidly extended to animal models[133] and then to human clinical trials (Table 12).[134,135] The trials in human beings have not yielded the expected lower restenosis rates. It is unlikely, though, that angiotensin is the only or even one of the major mitogenic factors contributing to smooth muscle cell activation.

Antagonists to Calcium Channels and β-Adrenergic Receptors

Increased coronary artery vasoreactivity (spasm) is one potential complication of coronary angioplasty. Coronary spasm has been documented both with diagnostic coronary arteriography and with coronary angioplasty. Spasm or elastic recoil of the vessel may play a role in restenosis after angioplasty, although together they probably account for far less than the 25% to 30% of restenosis suggested by some reports.[6] However, there is some evidence in experimental animal models that calcium-channel antagonists inhibit the formation of atherosclerotic-type lesions.[136] It was thus natural that calcium-channel antagonists should be considered for prevention of restenosis after angioplasty. Beta-adrenergic receptor antagonists also have antivasospastic properties, and they rightfully also should be considered. Several clinical trials and one review were conducted (Table 13).[137–142] The conclusions are conflicting, but overall the studies do not strongly support the contention that these agents are effective. Of three trials that evaluated the agent diltiazem, only one showed an effect; interestingly, however, this trial only recruited patients un-

TABLE 11. Studies of the Effects of Prostanoids and of Thromboxane and Serotonin Inhibitors on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Conclusion/comment
			Control	Treated	
Prostanoids					
Raizner et al.[122]	311	248	53	41(ciprostene)	Trend
DeServi et al.[123]	105	56	58	31(prostaglandin E_1)	Trend
Knudtson et al.[124]	270	250	32	27(prostacyclin)	Not effective
Thromboxane inhibitors					
Yabe et al.[125]	33	33	42	18	Trend
Finci et al.[126]	107	57	61	65	Not effective
Bove et al.[127]	755	415	MLD 1.43 mm	MLD 1.43 mm	Not effective
Serruys et al.[128]	697	522	MLD 1.46 mm	MLD 1.49 mm	Not effective
Feldman et al.[129]	1080	936	33	Low dose 25, high dose 26	Effective, but clinical event rates only reported
Serotonin inhibitors					
Klein et al.[130]	45	45	29	33	Not effective
Heik et al.[131]	97	86	38	22	Not effective

MLD, Minimum lumen diameter.

TABLE 12. Studies of the Effects of Angiotensin-Converting Enzyme Inhibitors on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Conclusion/comment
			Control	Treated	
Serruys et al.[134]	693	395	28	28 (cilazapril	Not effective
Desmet et al.[135]	180	75	51	42 (fosinopril	Not effective

dergoing their first coronary angioplasty. The other two diltiazem trials recruited both first-time and repeat patients. In the study by Hoberg et al.,[141] verapamil reduced restenosis in patients with stable angina but not in those with unstable angina. These points about first-time versus repeat angioplasty and stable angina versus unstable angina highlight once again the necessity for homogeneous patient groups for restenosis studies. An extensive review by Johansson et al.[142] found that restenosis rates were lower in patients taking calcium-channel blockers, β-blockers, or a combination of the two types of agents, but the differences did not reach significance.

Miscellaneous Agents

Several other categories of agents have been tested clinically for potential inhibition of restenosis (Table 14).[143–148] All have extensive logical reasoning behind their investigational use. None, however, have so far been any more effective in reducing restenosis than any other agents. Glucocorticosteroids have antiinflammatory and antiproliferative properties, but three trials of steroids did not find any reduction in restenosis rates. Lipid lowering has been advocated as an adjunct to coronary angioplasty for a variety of reasons. Many patients undergoing angioplasty have elevated baseline lipid levels, and this puts them at risk for further progression of their coronary disease. Clinical event rates certainly fall when lipid values decline, and there is some evidence for actual regression of established atherosclerosis with lipid lowering. Fish oils as dietary supplements are almost uniformly associated with lower restenosis rates, possibly with a statistically significant effect, and they may achieve this effect in part by lowering overall lipid amounts. Two trials of lipid-lowering agents produced opposing conclusions. A small trial by Hollman et al.[146] with the combination of lovastatin and colestipol found a restenosis rate of 34%, which they concluded was not different from historical values. A larger trial by Sahni et al.[147] documented a restenosis rate of 13% in patients randomly assigned to lovastatin and a significantly higher rate of 42% in patients randomly assigned to placebo. Although encouraging, the study was limited by the fact that only 55% of the treatment group and 37% of the control group had follow-up angiography. A larger and more complete clinical trial of lovastatin is planned.[150]

TABLE 13. Studies of the Effects of Calcium-Channel Antagonists and β-Adrenergic Receptor Antagonists on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Agent	Conclusion/comment
			Control	Treated		
Corcos et al.[137]	92	92	22	15	Diltiazem	Not effective
O'Keefe et al.[138]	201	120	32	36	Diltiazem	Not effective
Unverdorben et al.[139]	170	170	38	21	Diltiazem	Effective
Whitworth et al.[140]	241	198	30	28	Nifedipine	Not effective
Hoberg et al.[141]	196	172	62	56, unstable angina	Verapamil	Not effective
			63	38, stable angina	Verapamil	Effective
Johansson et al.[142]	541*	428*	50	—	No BB, CC;	Not effective
				31	BB, CC	
				34	No BB, CC	
				39	BB, CC	

BB, Any β-adrenergic blocking agent; *CC*, any calcium-channel antagonist.
*Number of procedures.

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TABLE 14. Studies of the Effects of Miscellaneous Agents on Restenosis After Coronary Angioplasty

Reference	Patients enrolled	Number with angiographic follow-up	Restenosis rates (%)		Agent	Conclusion/comment
			Control	Treated		
Steroids						
Stone et al.[143]	102	54	40	36	Prednisone	Not effective
Rose et al.[144]	66	58	33	33	Medrol	Not effective
Pepine et al.[145]	915	510	39	40	Prednisolone	Not effective
Lipid-lowering agents						
Hollman et al.[146]	55	44	—	34	Lovastatin + colestipol	Not effective
Sahni et all.[147]	157	79	42	13	Lovastatin	Effective
Anti-mitotic agents						
O'Keefe et al.[148]	197	145	22	22	Colchicine	Not effective
Thrombolytic agents						
Haine et al.[149]	199	166	38	33	Urokinase	Not effective

Colchicine is an antimitotic agent that inhibits spindle formation in dividing cells. In an experimental animal model, it was effective in preventing hyperplastic neointimal formation after balloon injury.[151] However, a large clinical trial failed to demonstrate any beneficial effects.[148]

Future Trials

The near future will see several more clinical trials evaluating newer agents for possible reduction in restenosis. A number of agents that more specifically inhibit platelets will be tested. These include several agents that block the platelet surface glycoprotein IIb/IIIa receptor (7E3 monoclonal antibody, peptides, and others), agents that inhibit von Willebrand factor, and agents that reduce actions of platelet activators (clopidogrel). Newer antithrombin anticoagulant agents are under investigation (hirudin, hirulog). Finally, specific and nonspecific inhibitors of growth factors may be tested. Angiopeptin, an analog of somatostatin, has nonspecific antagonism to several growth factors, including insulin-like growth factor and epidermal growth factor. Angiopeptin has been shown to inhibit myointimal proliferation in rat, rabbit, and primate models.[152] A clinical trial evaluating this agent is currently in progress. Inhibitors of or antibodies to various growth factors are under development, and some of these will no doubt eventually be tested clinically. It may also be possible someday to alter the genetic makeup of cells, specifically the arterial wall endothelial cells and smooth muscle cells, such that growth factors are not produced or are produced in inadequate amounts to send the signals for transformation and proliferation. This involves gene transfer into these target cells, a subject from the field of molecular biology now making its way into cardiology.[153] Several potential methods exist for transferring small fragments of genetic material, including direct absorption, liposome encapsulation, and retrovirus vectors. The future will bring many new approaches for altering the messages for biochemical products of cells.

MECHANICAL APPROACHES TO RESTENOSIS

Balloon dilatation angioplasty has been associated with substantial amounts of arterial injury and subsequent nontrivial rates of restenosis. This has sparked searches for various alternative forms of transluminal mechanical revascularization, undertaken in the hope that they would carry lower rates of restenosis. Atherectomy, or transluminal removal of atheromatous material, has the potential advantage over balloon dilatation of enlarging the arterial lumen with lesser degrees of residual stenosis because atheroma debulking is taking place. A smoother, less irregular lumen may be left behind. Directional coronary atherectomy, transluminal extraction endarterectomy, and rotational ablation are three alternative techniques developed to achieve this effect. The technical details of these procedures are beyond the scope of this review. The hope that these alternative procedures would lead to substantially lower restenosis rates has not been borne out. In fact, all forms of mechanical revascularization could be considered to be types of arterial injury, with restenosis a generalized response to that injury. From that perspective, one should not expect lower restenosis rates when these other techniques are used. With restenosis rates in the 30% to 60% range,[154–156] there does not appear to be any great advantage to these devices overall. However, for individual cases the goal is to achieve adequate initial lumen enlargement without complications. All of these devices may help achieve that goal when balloon dilatation may or does not. Limited clinical roles therefore exist for these methods, and these roles may expand in the future.

A limited clinical role will probably also exist for another mechanical device, the intracoronary stent. There are currently four different stent designs in use, all of which are investigational for coronary application at this time. Approval of one or more of these designs is expected in the near future. Stenting appears to produce acceptable (and possibly superior) initial angiographic and flow results when performed by experienced practitioners. After successful stent implantation, the coronary lumen is wider and the luminal borders are smoother than with conventional balloon angioplasty alone. This may or may not translate into lowered restenosis rates. A recent analysis of 250 stent procedures documented a restenosis rate of 25%, with stent placement into the left anterior descending coronary artery being the strongest correlate of restenosis.[157] With both stenting and atherectomy, there is evidence that the immediate postprocedure luminal diameter is the single most important determinant of later restenosis.[158] Within this analysis, it is the ability of stents and atherectomy to produce larger immediate gains in luminal diameter that accounts for any measurable differences in restenosis. Further experience and research will be required to refine these concepts. Stents can be made of various materials coated with various substances. This offers the possibility of making the stent the vehicle for localized drug delivery to the injured arterial wall. Clinical trials of coated stents likely will begin soon, and nonmetallic, polymeric stents are under development.

REFERENCES

1. ACC/AHA Task Force. Guidelines for percutaneous transluminal coronary angioplasty. J Am Coll Cardiol 1988;12:529–45.
2. Willerson JT, Hillis LD, Winniford M, Buja LM. Speculation regarding mechanisms responsible for acute ischemic heart disease syndromes. J Am Coll Cardiol 1986;8:245–50.

3. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. N Engl J Med 1992;326:242–50.
4. Rensing BJ, Hermans WRM, Beatt KJ, et al. Quantitative angiographic assessment of elastic recoil after percutaneous transluminal coronary angioplasty. Am J Cardiol 1990;66:1039–44.
5. Fischell TA, Nellessen U, Johnson DE, Ginsburg R. Endothelium-dependent arterial vasoconstriction after balloon angioplasty. Circulation 1989;79:899–910.
6. Waller BF, Pinkerton CA, Orr CM, Slack JD, VanTassel JW, Peters T. Restenosis 1 to 24 months after clinically successful coronary balloon angioplasty: a necropsy study of 20 patients. J Am Coll Cardiol 1991;17:58B–70B.
7. Rensing BJ, Hermans WR, Strauss BH, Serruys PW. Regional differences in elastic recoil after percutaneous transluminal coronary angioplasty: a quantitative angiographic study. J Am Coll Cardiol 1991;17:34B–8B.
8. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J Am Coll Cardiol 1991;17:758–69.
9. Schwartz RS, Holmes DR, Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol 1992;20:1284–93.
10. Pasternak RC, Baughman KL, Fallon JT, Block PC. Scanning electron microscopy after coronary transluminal angioplasty of normal canine coronary arteries. Am J Cardiol 1980;45:591–8.
11. Block PC, Baughman KL, Pasternak RC, Fallon JT. Transluminal angioplasty: correlation of morphologic and angiographic findings in an animal model. Circulation 1980;61:778–85.
12. Waller BF, Gorfinkel HJ, Rogers FJ, Kent KM, Roberts WC. Early and late morphologic changes in major epicardial coronary arteries after percutaneous transluminal coronary angioplasty. Am J Cardiol 1984;53:42C–7C.
13. Gravanis MB, Roubin GS. Histopathologic phenomena at the site of percutaneous transluminal coronary angioplasty. Hum Pathol 1989;20:477–85.
14. Casscells W. Migration of smooth muscle and endothelial cells: critical events in restenosis. Circulation 1992;86:723–9.
15. Liu MW, Roubin GS, King SB. Restenosis after coronary angioplasty: potential biologic determinants and role of intimal hyperplasia. Circulation 1989;79:1374–87.
16. Reidy MA, Schwartz SM. Endothelial regeneration: time course of intima changes after small defined injury of rat aortic endothelium. Lab Invest 1981;44:301–8.
17. Clowes AW, Clowes HM, Reidy MA. Kinetics of cellular proliferation after arterial injury: endothelial and smooth muscle growth in chronically denuded vessels. Lab Invest 1986;54:295–303.
18. Schwartz RS, Murphy JG, Edwards WD, et al. Coronary artery restenosis and the virginal membrane: smooth muscle cell proliferation and the intact internal elastic lamina. J Invasive Cardiol 1991;3:3–8.
19. Mizuno Y, Miyamoto A, Shibuya T, et al. Changes of angioscopic macromorphology following coronary angioplasty [abstract]. Circulation 1988;78:II-289.
20. Uchida Y, Hasegawa K, Kawamura K, Shibuya J. Angioscopic observation of the coronary luminal changes induced by coronary angioplasty. Am Heart J 1989;117:769–76.
21. Block P, Myler R, Stertzer S, Fallon J. Morphology after transluminal angioplasty in human beings. N Engl J Med 1981;305:382–5.

22. Waller BF. Morphologic correlates of coronary angiographic patterns at the site of coronary angioplasty. Clin Cardiol 1988;11:817–22.
23. Potkin BN, Roberts WC. Effects of coronary angioplasty on atherosclerotic plaques and relation of plaque composition and arterial size to outcome. Am J Cardiol 1988;62:41–50.
24. Ip JH, Fuster V, Badimon L, Badimon J, Taubman MB, Chesebro JH. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. J Am Coll Cardiol 1990;15:1667–87.
25. Garratt KN, Holmes DR, Bell MR, et al. Restenosis after directional coronary atherectomy: differences between primary atheromatous and restenosis lesions and influence of subintimal tissue resection. J Am Coll Cardiol 1990;16:1665–71.
26. Kuntz RE, Hinohara T, Safian RD, Selmon MR, Simpson JB, Baim DS. Restenosis after directional coronary atherectomy: effects of luminal diameter and deep wall excision. Circulation 1992;86:1394–9.
27. Leung DY, Glagov S, Mathews MB. Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro. Science 1976;191:475–7.
28. Saremback IJ, LaVeau PJ, Sigal SL, et al. Influence of inflation pressure and balloon size on the development of intimal hyperplasia after balloon angioplasty. Circulation 1989;80:1029–40.
29. Clowes AW, Clowes MM, Fingerle J, Reidy MA. Kinetics of cellular proliferation after arterial injury. V. Role of acute distension in the induction of smooth muscle cell proliferation. Lab Invest 1989;60:360–4.
30. Friedman RJ, Stemerman MB, Werz B, et al. The effect of thrombocytopenia on experimental arteriosclerotic lesion formation in rabbit. J Clin Invest 1977;60:1191–201.
31. Halon DA, Merdler A, Sheffer A, Flugelman MY, Lewis BS. Identifying patients at high risk for restenosis after percutaneous transluminal coronary angioplasty for unstable angina pectoris. Am J Cardiol 1989;64:289–93.
32. Hirshfeld JW, Schwartz JS, Jugo R, et al. Restenosis after coronary angioplasty: a multivariant statistical model to relate lesion and procedural variables to restenosis. J Am Coll Cardiol 1991;18:647–56.
33. Willerson JT, Yao SK, McNatt J, et al. Frequency and severity of cyclic flow alternations and platelet aggregation predict the severity of neointimal proliferation following experimental coronary stenosis and endothelial injury. Proc Natl Acad Sci U S A 1991;88:10624–8.
34. Ross R, Raines E, Bowen-Pope D. The biology of platelet-derived growth factor. Cell 1986;46:155–69.
35. Nemecek GM, Coughlin SR, Handley DA, Moskowitz MA. Stimulation of aortic smooth muscle cell mitogenesis by serotonin. Proc Natl Acad Sci U S A 1986;83:674–8.
36. Casscells W. Smooth muscle cell growth factors. Prog Growth Factor Res 1991;3:177–206.
37. Casscells W, Lappi DA, Olwin B, et al. Elimination of smooth muscle cells in experimental restenosis: targeting of FGF receptors. Proc Natl Acad Sci U S A 1992;89:7159–63.
38. Cercek B, Fishbein MC, Forrester JS, Helfant RH, Fagin JA. Induction of insulin-like growth factor-1 m-RNA in rat aorta after balloon denudation. Circ Res 1990;66:1755–60.
39. Loppnow H, Libby P. Proliferating or interleukin 1–activated human vascular smooth muscle cells secrete copious interleukin 6. J Clin Invest 1990;85:731–8.

40. Stiles CD, Capone GT, Scher CD, Antoniades HN, Van Wyk JJ, Pledger WJ. Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc Natl Acad Sci U S A 1979;76:1279–83.
41. Grunwald J, Haudenschild CC. Intimal injury in vivo activates vascular smooth muscle cell migration and explant outgrowth in vitro. Arteriosclerosis 1984;4:163–8.
42. Walker LN, Bowen-Pope DF, Ross R, Reidy MA. Production of platelet-derived growth factor–like molecules by cultured arterial smooth cells accompanies proliferation after arterial injury. Proc Natl Acad Sci U S A 1986;83:7311–5.
43. Cole CW, Hagen PO, Lucas JF, et al. Association of polymorphonuclear leukocytes with sites of aortic catheter-induced injury in rabbits. Atherosclerosis 1987;67:229–36.
44. Prescott MF, McBride CK, Court M. Development of intimal lesions after leukocyte migration into the vascular wall. Am J Pathol 1989;135:835–46.
45. Reidy MA. In vivo proliferation of vascular smooth muscle cells in vessels with intact endothelium (abstract). Fed Proc 1986;45:683.
46. Castellot JJ, Wright TC, Karnovsky MJ. Regulation of vascular smooth muscle cell growth by heparin and heparin sulfates. Semin Thromb Hemostasis 1987;13:489–503.
47. Wight TN. Cell biology of arterial proteoglycans. Arteriosclerosis 1989;9:1–20.
48. Assoian RK, Grotendorst GR, Miller, Sporn MB. Cellular transformation by coordinate action by three peptide growth factors from human platelets. Nature 1984;309:804–6.
49. Nathan C. Secretory products of macrophages. J Clin Invest 1987;79:319–26.
50. Clowes AW, Clowes MM, Au YPT, Reidy MA, Belin D. Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. Circ Res 1990;67:61–7.
51. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle cell growth in the absence of endothelium. Lab Invest 1983;49: 327–33.
52. Consigny PM, Tulenko TN, Nicosia RF. Immediate and long-term effects of angioplasty–balloon dilation on normal rabbit iliac artery. Arteriosclerosis 1986;6:265–76.
53. Stemerman MB, Colton C, Morrell E. Perturbations of the endothelium. Prog Hemost Thromb 1984;7:289–324.
54. Reidy MA, Standert D, Schwartz SM. Inhibition of endothelial cell regrowth: cessation of aortic endothelial cell replication after balloon catheter denudation. Arteriosclerosis 1982;2:216–20.
55. Reidy MA, Clowes AW, Schwartz SM. Endothelial regeneration: V. inhibition of endothelial regrowth in arteries of rat and rabbit. Lab Invest 1983;49:569–75.
56. Bobbio M, Detrano R, Colombo A, Lehmann KG, Park JB. Restenosis rate after percutaneous transluminal coronary angioplasty: a literature overview. J Invasive Cardiol 1991;3:214–24.
57. Serruys PW, Rensing BJ, Hermans WRM, Beatt KJ. Definition of restenosis after percutaneous transluminal coronary angioplasty: a quickly evolving concept. J Intervent Cardiol 1991;4:265–76.
58. Gruentzig AR, King SB, Schlumpf M, Siegenthaler W. Long-term follow-up after percutaneous transluminal coronary angioplasty: the early Zurich experience. N Engl J Med 1987;316:1127–32.
59. Simonton CA, Mark DB, Hinohara T, et al. Late restenosis after emergent coronary angioplasty for acute myocardial infarction: comparison with elective coronary angioplasty. J Am Coll Cardiol 1988;11:698–705.
60. Levine S, Ewels CJ, Rosing DR, Kent KM. Coronary angioplasty: clinical and angiographic follow-up. Am J Cardiol 1985;55:673–6.
61. Mabin TA, Holmes DR, Smith HC, et al. Follow-up clinical results in patients undergoing percutaneous transluminal coronary angioplasty. Circulation 1985;71:754–60.
62. Zaidi AR, Hollman J, Galan K, Belardi J, Franco I, Simpfendorfer C. Predictive value of chest discomfort for restenosis following successful coronary angioplasty [abstract]. Circulation 1985;72:III–456.
63. Holmes DR, Vlietstra RE, Smith HC, et al. Restenosis after percutaneous transluminal coronary angioplasty (PTCA): a report from the PTCA registry of the National Heart, Lung, and Blood Institute. Am J Cardiol 1984;53:77C–81C.
64. Bengtson JR, Mark DB, Honan MB, et al. Detection of restenosis after elective coronary angioplasty using the exercise treadmill test. Am J Cardiol 1990;65:28–34.
65. Califf RM, Ohman EM, Frid DJ, et al. Restenosis: the clinical issues. In: Topol EJ, ed. Textbook of interventional cardiology. Philadelphia: WB Saunders, 1990:363–4.
66. El-Tamimi H, Davies GJ, Hackett D, Fragasso G, Crea F, Maseri A. Very early prediction of restenosis after successful coronary angioplasty: anatomical and functional assessment. J Am Coll Cardiol 1990;15:259–64.
67. O'Keefe JH, Lapeyre AC, Holmes DR, Gibbons RJ. Usefulness of early radionuclide angiography for identifying low-risk patients for late restenosis after percutaneous transluminal coronary angioplasty. Am J Cardiol 1988;61:51–4.
68. Wijns W, Serruys PW, Reiber JHC, et al. Early detection of restenosis after successful percutaneous transluminal coronary angioplasty by exercise-redistribution thallium scintigraphy. Am J Cardiol 1985;55:357–61.
69. Wijns W, Serruys PW, Simoons ML, et al. Predictive value of early maximal exercise test and thallium scintigraphy after successful percutaneous transluminal angioplasty. Br Heart J 1985;53:194–200.
70. Scholl JM, Chaitman BR, David PR, et al. Exercise electrocardiography and myocardial scintigraphy in the serial evaluation of the results of percutaneous transluminal coronary angioplasty. Circulation 1982;66:380–90.
71. Ernst SMPG, Hillebrand FA, Klein B, Ascoop CA, van Tellingen C, Plokker HWM. The value of exercise tests in the follow-up of patients who underwent transluminal coronary angioplasty. Int J Cardiol 1985;7:267–79.
72. Rosing DR, van Raden MJ, Mincemoyer RM, et al. Exercise, electrocardiographic and functional responses after percutaneous transluminal coronary angioplasty. Am J Cardiol 1984;53:36C–41C.
73. Honan MB, Bengtson JR, Pryor DB, et al. Exercise treadmill testing is a poor predictor of anatomic restenosis after angioplasty for acute myocardial infarction. Circulation 1989;80:1585–94.
74. Pirelli S, Danzi GB, Alberti A, et al. Comparison of usefulness of high-dose dipyridamole echocardiography and exercise electrocardiography for detection of asymptomatic restenosis after coronary angioplasty. Am J Cardiol 1991;67:1335–8.
75. Hardoff R, Shefer A, Gips S, et al. Predicting late restenosis after coronary angioplasty by very early (12 to 24 h) thallium-201 scintigraphy: implications with regard to mechanisms of late coronary restenosis. J Am Coll Cardiol 1990;15:1486–92.

76. Jain A, Mahmarian JJ, Borges-Neto S, et al. Clinical significance of perfusion defects by thallium-201 single photon emission tomography following oral dipyridamole early after coronary angioplasty. J Am Coll Cardiol 1988;11:970–6.
77. Lam JYT, Chaitman BR, Byers S, et al. Can dipyridamole thallium imaging predict restenosis after coronary angioplasty? [abstract] Circulation 1987; 76:IV-373.
78. Hecht HS, Shaw RE, Bruce TR, Ryan C, Stertzer SH, Myler RK. Usefulness of tomographic thallium-201 imaging for detection of restenosis after percutaneous transluminal coronary angioplasty. Am J Cardiol 1990;66:1314–8.
79. Manyari DE, Knudtson M, Kloiber R, Roth D. Sequential thallium-201 myocardial perfusion studies after successful percutaneous transluminal coronary angioplasty: delayed resolution of exercise-induced scintigraphic abnormalities. Circulation 1988;77:86–95.
80. El-Tamimi H, Davies GJ, Sritara P, Hackett D, Crea F, Maseri A. Inappropriate constriction of small coronary vessels as a possible cause of a positive exercise test early after successful coronary angioplasty. Circulation 1991;84:2307–12.
81. Kipfer PH, Hess OM, Gaglione A, Krayenbuehl HP. Exercise-induced ST-segment depression after successful PTCA: incomplete revascularization or altered coronary vasomotor tone? Coronary Artery Dis 1991;2:595–604.
82. Nobuyoshi M, Kimura T, Nosaka H, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: serial angiographic follow-up of 229 patients. J Am Coll Cardiol 1988;12:616–23.
83. Serruys PW, Luijten HE, Beatt KJ, et al. Incidence of restenosis after successful coronary angioplasty: a time-related phenomenon. Circulation 1988;77:361–71.
84. Fanelli C, Aronoff R. Restenosis following coronary angioplasty. Am Heart J 1990;119:357–68.
85. Hermans WRM, Rensing BJ, Strauss BH, Serruys PW. Prevention of restenosis after percutaneous transluminal coronary angioplasty: the search for a 'magic bullet.' Am Heart J 1991;122:171–87.
86. Leimgruber PP, Roubin GS, Anderson HV, et al. Influence of intimal dissection on restenosis after successful coronary angioplasty. Circulation 1985;72:530–5.
87. Matthews BJ, Ewels CJ, Kent KM. Coronary dissection: a predictor of restenosis? Am Heart J 1988;115:547–54.
88. Hermans WRM, Rensing BJ, Foley DP, et al. Therapeutic dissection after successful coronary balloon angioplasty: no influence on restenosis or on clinical outcome in 693 patients. J Am Coll Cardiol 1992;20:767–80.
89. Califf RM, Fortin DF, Frid DJ, et al. Restenosis after coronary angioplasty: an overview. J Am Coll Cardiol 1991;17:2B–13B.
90. Barnathan ES, Schwartz JS, Taylor L, et al. Aspirin and dipyridamole in the prevention of acute coronary thrombosis complicating coronary angioplasty. Circulation 1987;76:125–34.
91. Lembo NJ, Black AJR, Roubin GS, et al. Effect of pretreatment with aspirin versus aspirin plus dipyridamole on frequency and type of acute complications of percutaneous transluminal coronary angioplasty. Am J Cardiol 1990;65:422–6.
92. White CW, Knudson M, Schmidt D, et al. Neither ticlopidine nor aspirin-dipyridamole prevents restenosis post PTCA: results from a randomized placebo-controlled multicenter trial [abstract]. Circulation 1987;76:IV-213.
93. Ellis SG, Roubin GS, Wilentz J, Lin S, Douglas JS, King SB 3d. Results of a randomized trial of heparin and aspirin vs aspirin alone for prevention of acute closure and restenosis after angioplasty [abstract]. Circulation 1987;76:IV-213.
94. Schanzenbacher P, Grimmer M, Maisch B, Kochsiek K. Effect of high dose and low dose aspirin on restenosis after primary successful angioplasty [abstract]. Circulation 1988;78:II-99.
95. Mufson L, Black A, Roubin G, et al. A randomized trial of aspirin in PTCA: effect of high vs low dose aspirin on major complications and restenosis [abstract]. J Am Coll Cardiol 1988;11:236A.
96. Schwartz L, Bourassa MG, Lesperance J, et al. Aspirin and dipyridamole in the prevention of restenosis after percutaneous transluminal coronary angioplasty. N Engl J Med 1988;318:1714–9.
97. Dyckmans J, Thonnes W, Ozbek C, et al. High vs low dosage of acetylic salicylic acid for prevention of restenosis after successful PTCA: preliminary results of a randomized trial [abstract]. Eur Heart J 1988;9[Suppl 1):58.
98. Finci L, Meier B, Steffenino G, Rutishauser W. Aspirin versus placebo after coronary angioplasty for prevention of restenosis [abstract]. Eur Heart J 1988;9(Suppl 1):156.
99. Schwartz L, Lesperance J, Bourassa MG, et al. The role of antiplatelet agents in modifying the extent of restenosis following percutaneous transluminal coronary angioplasty. Am Heart J 1990;119:232–6.
100. Taylor BR, Gibbons FA, Cope GD, Cumpston GN, Mews GC, Luke P. Effects of low-dose aspirin on restenosis after coronary angioplasty. Am J Cardiol 1991;68:874–8.
101. Bertrand ME, Allain H, Lablanche JM. Results of a randomized trial of ticlopidine versus placebo for prevention of acute closure and restenosis after coronary angioplasty (PTCA): the TACT study [abstract]. Circulation 1990; 82:III-190.
102. Kitazume H, Kubo I, Iwama T, Ageishi Y, Suzuki A. Combined use of aspirin, ticlopidine and nicorandil prevented restenosis after coronary angioplasty [abstract]. Circulation 1988;78:II-633.
103. Liu MW, Roubin GS, Robinson KA, et al. Trapidil in preventing restenosis after balloon angioplasty in the atherosclerotic rabbit. Circulation 1990;81:1089–93.
104. Maresta A, Balducelli M, Cantini L, et al. Trapidil (PDGF antagonist) vs ASA in the prevention of restenosis after PTCA: a double blind randomized trial [abstract]. J Am Coll Cardiol 1992;19:170A.
105. Nishikawa H, Ono N, Motoyasu M, et al. Preventive effects of trapidil (PDGF antagonist) on restenosis after PTCA [abstract]. Circulation 1992;86:I-53.
106. Okamoto S, Inden M, Setsuda M, Konishi T, Nakano T. Effects of trapidil (triazolopyrimidine), a platelet-derived growth factor antagonist, in preventing restenosis after percutaneous transluminal coronary angioplasty. Am Heart J 1992;123:1439–44.
107. Thornton MA, Gruentzig AR, Hollman J, King SB, Douglas JS. Coumadin and aspirin in prevention of restenosis after transluminal coronary angioplasty: a randomized study. Circulation 1984;69:721–7.
108. Urban P, Buller N, Fox K, Shapiro L, Bayliss J, Rickards A. Lack of effect of warfarin on the restenosis rate or on clinical outcome after balloon coronary angioplasty. Br Heart J 1988;60:485–8.
109. Ellis SG, Roubin GS, Wilentz J, Douglas JS, King SB. Effect of 18- to 24-hour heparin administration for prevention of restenosis after uncomplicated coronary angioplasty. Am Heart J 1989;117:777–82.
110. Hirshfeld JW, Goldberg S, MacDonald R, et al. Lesion and procedure-related

variables predictive of restenosis after PTCA—a report from the M-HEART study group [abstract]. Circulation 1987;76:IV-215.
111. Hanke H, Oberhoff M, Hanke S, et al. Inhibition of cellular proliferation after experimental balloon angioplasty by low-molecular-weight heparin. Circulation 1992;85:1548–56.
112. Faxon DP, Spiro T, Minor S, et al. Enoxaparin, a low molecular weight heparin, in the prevention of restenosis after angioplasty: results of a double blind randomized trial [abstract]. J Am Coll Cardiol 1992;19:258A.
113. Fox PL, DiCorleto PR. Fish oils inhibit endothelial cell production of platelet-derived growth factor–like protein. Science 1988;241:453–6.
114. Slack JD, Pinkerton CA, VanTassel J, Orr CM, Nasser WK. Can oral fish oil supplement minimize re-stenosis after percutaneous transluminal coronary angioplasty [abstract]? J Am Coll Cardiol 1987;9:64A.
115. Dehmer GJ, Popma JJ, Van Den Berg EK, et al. Reduction in the rate of early restenosis after coronary angioplasty by a diet supplemented with n-3 fatty acids. N Engl J Med 1988;319:733–40.
116. Milner MR, Gallino RA, Leffingwell A, Pichard AD, Brooks-Robinson S, Rosenberg J, Little T, Lindsay J. Usefulness of fish oil supplements in preventing clinical evidence of restenosis after percutaneous transluminal coronary angioplasty. Am J Cardiol 1989;64:294–9.
117. Grigg LE, Kay TWH, Valentine PA, et al. Determinants of restenosis and lack of effect of dietary supplementation with eicosapentaenoic acid on the incidence of coronary artery restenosis after angioplasty. J Am Coll Cardiol 1989;13:665–72.
118. Reis GJ, Boucher TM, Sipperly ME, et al. Randomised trial of fish oil for prevention of restenosis after coronary angioplasty. Lancet 1989;2:177–81.
119. Olsson AG. Fiskolja forhindrar restenos av kranskarlsangioplastik. Lakartidningen 1989;86:1051–2.
120. Nye ER, Ablett MB, Robertson MC, Ilsley CDJ, Sutherland WHF. Effect of eicosapentaenoic acid on restenosis rate, clinical course and blood lipids in patients after percutaneous transluminal coronary angioplasty. Aust N Z J Med 1990;20:549–52.
121. Bairati I, Roy L, Meyer F. Double-blind, randomized, controlled trial of fish oil supplements in prevention of recurrence of stenosis after coronary angioplasty. Circulation 1992;85:950–6.
122. Raizner A, Hollman J, Demke D, Wakefield L. Beneficial effects of ciprostene in PTCA: a multicenter, randomized, controlled trial [abstract]. Circulation 1988;78:II-290.
123. DeServi S, Klugman S, Chioin R, Barbieri E, Sperandeo A. Prostaglandin E1 administration in unstable angina patients undergoing PTCA: preliminary results [Abstr]. Eur Heart J 1990;11(Suppl):378.
124. Knudtson ML, Flintoft VF, Roth DL, Hansen JL, Duff HJ. Effect of short-term prostacyclin administration on restenosis after percutaneous transluminal coronary angioplasty. J Am Coll Cardiol 1990;15:691–7.
125. Yabe Y, Okamoto K, Oosawa H, et al. Does a thromboxane A2 synthetase inhibitor prevent restenosis after PTCA [abstract]? Circulation 1989;80:II-260.
126. Finci L, Hofling B, Ludwig B, et al. Sulotroban during and after coronary angioplasty. a double-blind, placebo controlled study. Z Kardiol 1989;78(Suppl 3):50–4.
127. Bove A, Savage M, Deutsch E, et al. Effects of selective and non-selective thromboxane A2 blockade on restenosis after PTCA: M-HEART II [abstract]. J Am Coll Cardiol 1992;19:259A.
128. Serruys PW, Rutsch W, Heyndrickx, et al. Prevention of restenosis after percutaneous transluminal coronary angioplasty with thromboxane A2-receptor blockade: a randomized, double-blind, placebo-controlled trial. Circulation 1991;84:1568–80.
129. Feldman RL, Bengtson JR, Pryor DB, Zimmerman MB. The GRASP study: use of a thromboxane A2 receptor blocker to reduce adverse clinical events after coronary angioplasty [abstract]. J Am Coll Cardiol 1992;19:259A.
130. Klein W, Eber B, Fluch N, Dusleag J. Ketanserin prevents acute occlusion but not restenosis after PTCA [abstract]. J Am Coll Cardiol 1989;13:44A.
131. Heik SCW, Bracht M, Benn HP, Erlemeier HH, Kupper W. No prevention of restenosis after PTCA with ketanserin. a controlled prospective randomized double-blind study [abstract]. Circulation 1992;86:I-53.
132. Powell JS, Muller RKM, Baumgartner HR. Suppression of the vascular response to injury: the role of angiotensin-converting enzyme inhibitors. J Am Coll Cardiol 1991;17:137B–42B.
133. Powell JS, Clozel JP, Muller RKM, et al. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Science 1989;245:186–8.
134. The Multicenter European Research Trial With Cilazapril After-Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MERCATOR) Study Group. Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? Results of the MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. Circulation 1992;86:100–10.
135. Desmet WJ, Vrolix MC, De Scheerder IK, Van Lierde JM, Piessens JH. Fosinopril sodium in restenosis prevention after coronary angioplasty [abstract]. Circulation 1992;86:I-54.
136. Henry PD, Bentley KI. Suppression of atherogenesis in cholesterol-fed rabbits treated with nifedipine. J Clin Invest 1981;68:1386–9.
137. Corcos T, David PR, Val PG, et al. Failure of diltiazem to prevent restenosis after percutaneous transluminal coronary angioplasty. Am Heart J 1985;109:926–31.
138. O'Keefe JH, Giorgi LV, Hartzler GO, Get al. Effects of diltiazem on complications and restenosis after coronary angioplasty. Am J Cardiol 1991;67:373–6.
139. Unverdorben M, Kunkel B, Leucht M, Bachmann K. Reduction of restenosis after PTCA by diltiazem? [abstract] Circulation 1992;86:I-53.
140. Whitworth HB, Roubin GS, Hollman J, et al. Effect of nifedipine on recurrent stenosis after percutaneous transluminal coronary angioplasty. J Am Coll Cardiol 1986;8:1271–6.
141. Hoberg E, Schwarz F, Schomig A, et al. Prevention of restenosis by verapamil: the verapamil angioplasty study (VAS) [abstract]. Circulation 1990;82:III-428.
142. Johansson SR, Lamm C, Bondjers G, Emanuelsson H, Hjalmarson A. Role of beta-adrenergic blockers after percutaneous transluminal coronary angioplasty. Am J Cardiol 1990;66: 915–20.
143. Stone GW, Rutherford BD, McConahay, et al. A randomized trial of corticosteroids for the prevention of restenosis in 102 patients undergoing repeat coronary angioplasty. Cathet Cardiovasc Diagn 1989;18:227–31.
144. Rose TE, Beauchamp BG. Short term, high dose steroid treatment to prevent restenosis in PTCA [abstract]. Circulation 1987;76:IV-371.
145. Pepine CJ, Hirshfeld JW, MacDonald RG, et al. A controlled trial of corticosteroids to prevent restenosis after coronary angioplasty. Circulation 1990;81:1753–61.
146. Hollman J, Konrad K, Raymond R, Whitlow P, Michalak M, Van Lente F. Lipid

lowering for the prevention of recurrent stenosis following coronary angioplasty [abstract]. Circulation 1989;80:II-65.
147. Sahni R, Manlet AR, Voci G, Banka V. Prevention of restenosis by lovastatin after successful coronary angioplasty. Am Heart J 1991;121:1600–8.
148. O'Keefe JH, McCallister BD, Bateman TM, Kuhnlein DL, Ligon RW, Hartzler GO. Ineffectiveness of colchicine for the prevention of restenosis after coronary angioplasty. J Am Coll Cardiol 1992;19:1597–600.
149. Haine E, Urban P, Dorsaz PA, Favre J, Meier B. Double-blind randomized evaluation of urokinase prior to angioplasty: early and late outcome [abstract]. Circulation 1992;86:I-652.
150. Weintraub WS, Bocuzzi SJ, et al. Background and methods for the lovastatin restenosis trial after percutaneous transluminal coronary angioplasty. Am J Cardiol 1992;70:293–9.
151. Currier JW, Pow TK, Minihan AC, Haudenschild CC, Faxon DP, Ryan TJ. Colchicine inhibits restenosis after iliac angioplasty in the atherosclerotic rabbit [abstract]. Circulation 1989;80:II-66.
152. Lundergan CF, Foegh ML, Ramwell PW. Peptide inhibition of myointimal proliferation by angiopeptin, a somatostatin analogue. J Am Coll Cardiol 1991;17:132B–6B.
153. Nabel EG, Plautz G, Nabel GJ. Gene transfer into vascular cells. J Am Coll Cardiol 1991;17:189B–94B.
154. Hinohara T, Robertson GC, Selmon MR, et al. Restenosis after directional coronary atherectomy. J Am Coll Cardiol 1992;20:623–32.
155. Sketch MH, O'Neill WW, Galichia JP, et al. The Duke multicenter coronary transluminal extraction-endarterectomy registry: acute and chronic results [abstract]. J Am Coll Cardiol 1991;17:31A.
156. Teirstein PS, Warth DC, Haq N, et al. High speed rotational coronary atherectomy for patients with diffuse coronary artery disease. J Am Coll Cardiol 1991;18:1694–701.
157. Carroza JP, Kuntz RE, Levine MJ, et al. Angiographic and clinical outcome of intracoronary stenting: immediate and long-term results from a large single-center experience. J Am Coll Cardiol 1992;20:328–37.
158. Kuntz RE, Safian RD, Carrozza JP, Fishman RF, Mansour M, Baim DS. The importance of acute luminal diameter in determining restenosis after coronary atherectomy or stenting. Circulation 1992;86:1827–35.

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The β3 Integrin Antagonist m7E3 Reduces Matrix Metalloproteinase Activity and Smooth Muscle Cell Migration

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Key Words

Smooth muscle cell · Matrix metalloproteinase · m7E3 · αvβ3 integrin · Arterial injury

Abstract

Treatment with c7E3 (abciximab, ReoPro) has been associated with a reduction in coronary events and the need for revascularization. Some of these beneficial effects may be due to blockade of the αvβ3 integrin receptor on smooth muscle cells (SMCs), however very little is known about the mechanisms involved. The current studies were designed to test the hypothesis that β3 integrin antagonists inhibit the arterial response to injury by reducing matrix metalloproteinase (MMP) activity in the vessel wall. Male Sprague-Dawley rats were treated with daily intraperitoneal injections of the monoclonal antibody m7E3 at a dose of 6 mg/kg/day. MMP-9 activity was reduced by 73%, and MMP-2 activity by 75%, in the injured carotids of the m7E3-treated rats compared to saline-treated controls. By contrast, tissue inhibitor metalloproteinase (TIMP) activity was not changed. SMC migration assayed at 4 days after injury was reduced from 56.7 ± 14 cells/mm² intimal surface area in controls to 17.5 ± 5 cells/mm² in m7E3-treated rats ($p = 0.02$). Medial cell replication measured at 4 days and intimal cell replication measured at 7 days were not affected. Intimal cross-sectional area, measured 14 days after injury was reduced by 28% after m7E3 treatment ($p = 0.05$). Intimal smooth muscle cell number and the ratio of intima/media cross-section area were also reduced. By contrast, intimal SMC density was not affected by m7E3 treatment, indicating no effect on matrix accumulation. We conclude that treatment with m7E3 reduced SMC migration following vascular injury, possibly via an inhibitory effect on MMP activity, and this resulted in a decrease in intimal size at 14 days after injury.

Treatment with c7E3 (abciximab, ReoPro) results in a reduction in coronary events and need for revascularization, with benefits extending long beyond the initial treatment period [1]. Although the antibody c7E3 Fab was developed to block glycoprotein αIIb/β3 receptors on platelets, and is used for its antithrombotic properties, it cross-reacts with other receptors including the αvβ3 integrin present on the surface of vascular smooth muscle cells (SMCs) and endothelial cells [2], thus part of its beneficial effects may be due to αvβ3 blockade. The αvβ3 integrin and several matrix ligands are upregulated in human atherosclerotic plaques and following experimen-

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tal arterial injury [3]. This integrin may play an important pathophysiologic role, since results from animal studies indicate that αvβ3 integrin blockade inhibits neointimal formation after angioplasty [4–10]. The mechanisms involved are not entirely clear; however studies from our lab and others have shown that αvβ3 blockade results in decreased SMC migration from media to intima [8,11], increased cell death by apoptosis [9], and decreased macrophage infiltration [12].

SMC migration from the media to the intima is facilitated by the activity of matrix metalloproteinases (MMPs) which are upregulated following arterial injury [13]. Recent studies including our own have implicated αvβ3 in the control of MMP expression and activity by vascular cells in tissue culture. We have shown that osteopontin stimulates MMP-9 and MMP-1 expression in SMCs via the αvβ3 integrin receptor [11]. Kanda et al. [14] have shown that αvβ3 is necessary to enable SMC proteolysis of, and invasion through a collagen lattice. Finally, αvβ3 binds active MMP-2 in endothelial cells, allowing localization of proteolytic activity to the leading edge of the migrating cell [15]. Although this work provides important evidence, all of these studies were done in tissue culture and it is not known whether αvβ3 plays a role in regulating MMP activity in vivo. In the current studies we hypothesize that αvβ3 integrin antagonists inhibit SMC migration by reducing MMP expression and activity following arterial injury.

We have used the monoclonal antibody m7E3 F(ab')$_2$ (m7E3) (the parent antibody for abciximab or ReoPro) which binds to rat β3 integrin receptors [16], to block SMC and platelet β3 receptors following balloon catheter injury of the rat carotid artery. The response to injury in the rat model is characterized by adhesion of a platelet monolayer to the vessel wall within 8 h, followed by proliferation of medial SMCs which peaks at 2 days after injury, and migration of SMCs from the media to the intima, with the first SMCs appearing in the intima 3–4 days after injury. Intimal SMCs undergo continued proliferation and synthesize an abundant extracellular matrix, with maximum intimal thickening evident 2–3 weeks following injury [17].

This study shows for the first time that treatment with m7E3 decreased MMP-9 and MMP-2 activity in the injured rat carotid artery and SMC migration from the media to the intima was also reduced. By contrast, SMC proliferation was not affected by m7E3. Importantly, there was a significant decrease in intimal cross-sectional area 2 weeks after injury in the m7E3-treated rats.

Materials and Methods

All chemicals were obtained from Sigma (St. Louis, Mo., USA) unless stated otherwise.

Surgery and m7E3 Treatment

Animal experiments were carried out in accordance with the guidelines of the Canada Council on Animal Care. A total of 68 male Sprague-Dawley rats (3–4 months old) (Charles River, Constant, Que., Canada) were used in all experiments. Balloon catheter injury of the left common carotid artery was performed as previously described [18]. Rats were anesthetized by intraperitoneal injection of xylazine (Rompun, 4.6 mg/kg body weight, Bayer Inc., Etobicoke Ont., Canada) and ketamine (Ketaset, 70 mg/kg body weight, Ayerst Veterinarian Laboratories, Guelph Ont., Canada). The left carotid artery was injured with a 2F balloon catheter (Baxter, Toronto, Ont., Canada) which was inflated and withdrawn three times to denude the vessel of endothelium. m7E3 F(ab')$_2$ at a dose of 6 mg/kg body weight was administered once a day by intraperitoneal injection, starting 18 h before balloon catheter injury, and continuing until sacrifice at 4, 7 or 14 days after injury. Control rats were injected with saline vehicle. Blood samples were taken by occipital bleed 24 h after balloon catheter injury to determine platelet counts.

To label all SMCs entering S-phase, a 50 mg pellet of 5-bromo-2'-deoxyuridine (BrdU; Boehringer Mannheim Corp., Laval, Que., Canada) was implanted subcutaneously at the nape of the neck in rats 24 h before sacrifice (4 and 7 day groups). The rats were killed by i.v. injection of T-61 which contains in 1 ml: 200 mg of N-[2-(m-methoxyphenyl)-2 ethylybutyl-(1)]γ hydroxy-butyramide, 50 mg of 4,4'-methylene-bis-(cyclohexyl, trimethylammonium iodide) and 5 mg of tetracaine hydrochloride with dimethyl formamide (Hoechst Roussel Veterinarian, Regina, Sask., Canada). The carotids were flushed with Ringer's solution (Baxter), then perfusion-fixed at physiologic pressure with 0.1 mol/l phosphate-buffered 4% paraformaldehyde. In the 4 day group, a 1 cm length was excised from the middle of the common carotid artery and used for the SMC migration assay as previously described [19]. Briefly, the intimal SMCs on the vessel surface were stained with an antibody against histone H1 (MAB1276, Chemicon Inc., Temecula, Calif., USA). The intimal surface was visualized by light microscopy, and the number of nuclei per square millimeter of intimal surface area was determined using a computerized image analysis system (Simple Image Analysis Software, Compix Inc., Mars, Pa., USA) In the 4-, 7- and 14-day groups, two-vessel segments 5 mm in length were cut 1 and 2 cm distal to the origin of the common carotid artery, embedded in paraffin, and cross-sections were cut and used for morphometry and BrdU labeling. Values obtained from these two sections were averaged to control for variability along the length of the vessel. SMC replication rates were determined at 4 and 7 days after injury by counting the number of BrdU-positive SMC nuclei, and expressing this as a percentage of total SMC nuclei in either medial or intimal layers of the cross-sections. Intimal and medial cross-sectional areas, the ratio of intimal:medial areas, total SMC number in the intima and media, and lumen area and external elastic lamina perimeter were measured in arteries from the 14-day group using computerized digital morphometry with Simple Image Analysis Software. Intimal and medial SMC densities were calculated dividing total SMC number by cross-sectional area.

Platelet Deposition

Platelet deposition was measured 48 h after balloon injury using scanning electron microscopy as we have previously described [11]. Platelet deposition was quantitated by counting the number of platelets per field in 5 nonoverlapping fields (magnification ×2,000) for each carotid. Two carotids per treatment group were analyzed.

Zymography

MMP-2 and 9 activity in arterial extracts taken 4 days after injury were determined by gelatin zymography as described in our previous publication [19]. The uninjured right carotids from each animal were used as controls. The gels were normalized by adding equal amounts of total protein extract to each lane. The MMPs on the gelatin zymograms were identified by their molecular weights, and by inhibition with EDTA or phenanthroline. Activity on the zymograms was quantified by scanning densitometric analysis of the bands using a Bio-Rad Gel 1000 documentation system and Molecular Analyst software (Biorad). Activity for each MMP in the injured carotids was expressed as the fold increase over activity in the uninjured control carotids.

Tissue inhibitors of metalloproteinases (TIMPs) were visualized by reverse zymography. Recombinant MMP-2 (Chemicon Inc.) was incorporated into a gelatin-containing polyacrylamide zymogram gel at a concentration of 0.13 μg/ml. Electrophoresis of arterial extracts was performed, then the gels were washed in 2.5% Triton X-100, incubated overnight in zymogram incubation buffer, then stained with Coomassie blue dye. TIMP activity was visualized as undigested bands remaining against a clear background. TIMP activity on the reverse zymogram was quantitated by scanning densitometry, and activity for injured carotids was expressed as the fold increase in activity over uninjured carotids.

Statistical Analysis

Values are expressed as mean ± SEM. Group means were compared by the two-tailed Student t test for independent samples.

Results

m7E3 Treatment Did Not Inhibit Platelet Deposition

Since m7E3 binds to platelet β3 integrins, and some intact antiplatelet antibodies induce thrombocytopenia, we measured plasma platelet counts in blood samples taken 24 h after balloon injury. There was no significant difference in platelet number between control (814 ± 31 × 10^9/l; n = 9) and m7E3-treated rats (941 ± 48 × 10^9/l; n = 8) (p = 0.11). The lack of thrombocytopenia was likely due to the lack of an Fc domain in the m7E3 F(ab')$_2$ antibody fragment. m7E3 treatment did not significantly affect platelet deposition on the vessel wall 48 h after injury. There were 466 ± 24 platelets/2,000× field in control animals and 391 ± 57 platelets/2,000× field in m7E3-treated rats (p = 0.177).

m7E3 Treatment Inhibited SMC Migration and MMP Production in Injured Rat Carotid Arteries

Migration from media to intima was assessed by staining the SMC nuclei and counting the nuclei in the intima 4 days after balloon catheter injury. SMC migration was decreased by 69% after m7E3 treatment, from 56.7 ± 14 cells/mm^2 in controls to 17.5 ± 5 cells/mm^2 in m7E3-treated rats (p = 0.02) (fig. 1a, b). m7E3 was deposited on the luminal surface of the carotids in treated rats, and it stained with the secondary antibody used in this procedure, resulting in the appearance of particulate matter on the surface of the vessels (fig. 1b). Rats treated with a control monoclonal antibody showed SMC migration similar to saline controls, and also showed particulate staining on the vessel surface (not shown).

MMP activity in carotid extracts was analyzed using gelatin zymography (fig. 2a). Several prominent bands were evident with molecular weights of 105, 88, 83, and 62 kD, and a doublet of 72 to 70 kD. A faint band with a molecular weight of 37 kD was present in some but not all samples. By comparison with Western blots using anti-MMP antibodies, and comparison with previous studies by ourselves and others, we have determined that the 105- and 88-kD bands correspond to latent and active MMP-9, respectively. The 72- to 70-kD doublet, and the 62-kD band correspond to latent (doublet) and active MMP-2, respectively. The identity of the 83-kD, and the 37-kD bands is unknown. In the presence of SDS in the sample buffer and the gels, zymogen forms of the MMPs undergo a conformational change which exposes the active site of the enzyme, enabling the zymogen to digest substrate.

There were no significant differences in MMP activity in uninjured carotids from saline- or m7E3-treated rats (data not shown). In saline-treated rats, the activity in all MMP bands was increased after balloon catheter injury compared to uninjured control arteries (fig. 2a). After m7E3 treatment, proteinase activity was decreased compared to injured, saline-treated rats (fig. 2a). Scanning densitometric analysis of the zymograms was performed and activity was expressed as the fold increase over activity in the saline-treated uninjured vessels (fig. 2b). The decreases in the MMP-9 latent (85%), MMP-9 active (73%), and MMP-2 active (75%) bands were statistically significant.

Three bands were evident on reverse zymograms from uninjured carotid arteries, with MW ~29, 28 and 21 kD (fig. 2c, lane 1) After injury, bands of ~31, 24 and 18 kD appeared (fig. 2c, lane 2). Based on previous studies and Western blots for TIMP-1 and TIMP-2, the 28- to 31-kD bands represent differently glycosylated forms of TIMP-1,

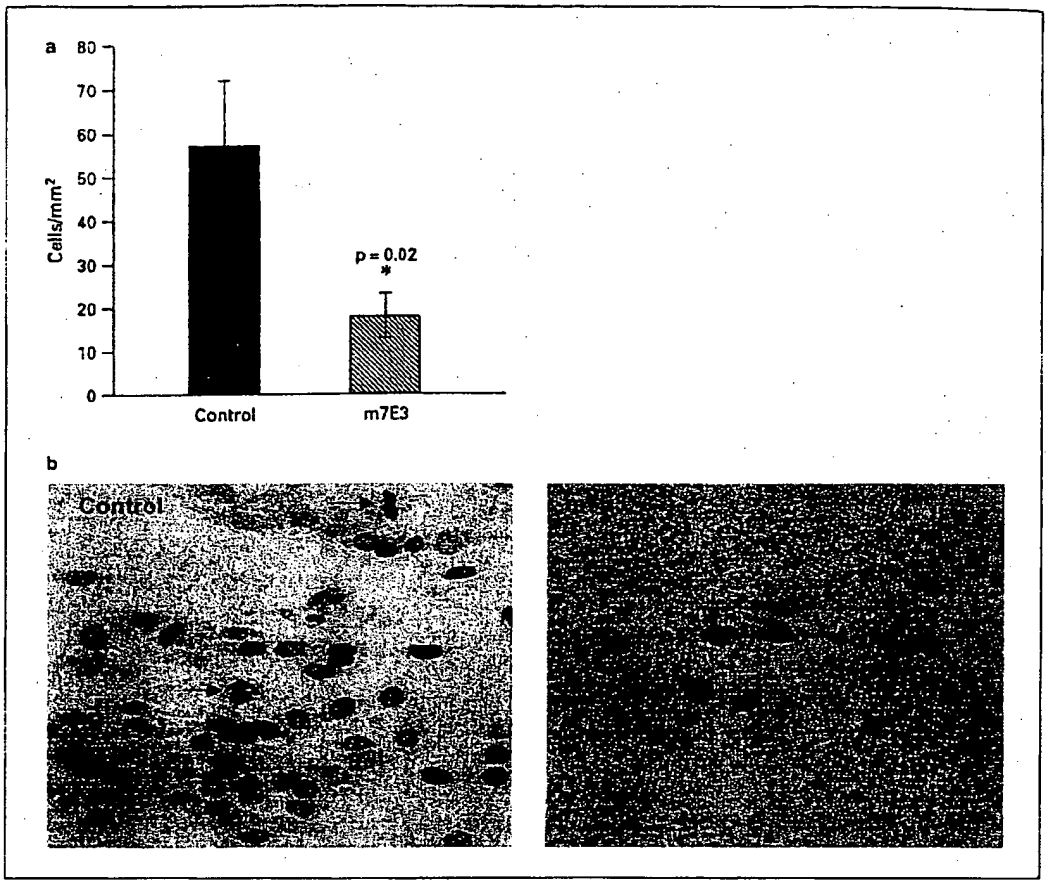

Fig. 1. SMC migration 4 days after balloon catheter injury of the carotid artery was significantly decreased in m7E3-treated rats. **a** Migration was measured by staining the SMCs on the intimal surface of the carotids with an anti-histone antibody and counting the number of cell nuclei per square millimeter intimal surface area. Values are mean ± SEM; n = 8 for m7E3-treated rats, and n = 9 for saline-treated control rats. **b** Representative photographs of SMCs on the intimal surface of control and m7E3-treated rats stained with monclonal antibody against histone to label the nuclei. Note the presence of mitotic figures in the control vessels (arrows). × 600.

and the 18- and 21-kD bands are TIMP-2. The identity of the 24-kD band is unknown, but may represent TIMP-3; however, no antibodies are available to rat TIMP-3 to confirm this. TIMP-1 activity was slightly increased after arterial injury, but TIMP-2 activity was not changed (fig. 2c, d). Treatment with m7E3 did not affect the activity of TIMP-1 or TIMP-2 (fig. 2d).

Medial and Intimal SMC Proliferation Were Not Affected by m7E3 Treatment

SMC replication rates were measured in the media at 4 days, and the neointima at 7 days following balloon injury. Medial SMC replication rate at 4 days after injury was 3.17 ± 0.89% in control rats, not significantly different from 4.2 ± 1.0% in m7E3-treated rats (p = 0.41) (fig. 3a). Intimal replication was measured at 7 days, and was not significantly different between control and treated rats (p = 0.21) (fig. 3b).

Intimal Area and SMC Number Was Decreased, and Lumen Area Increased after m7E3 Treatment

Rats were treated with m7E3 or saline for 14 days after left carotid balloon injury, then morphometric measurements were made on tissue cross-sections. There was a sig-

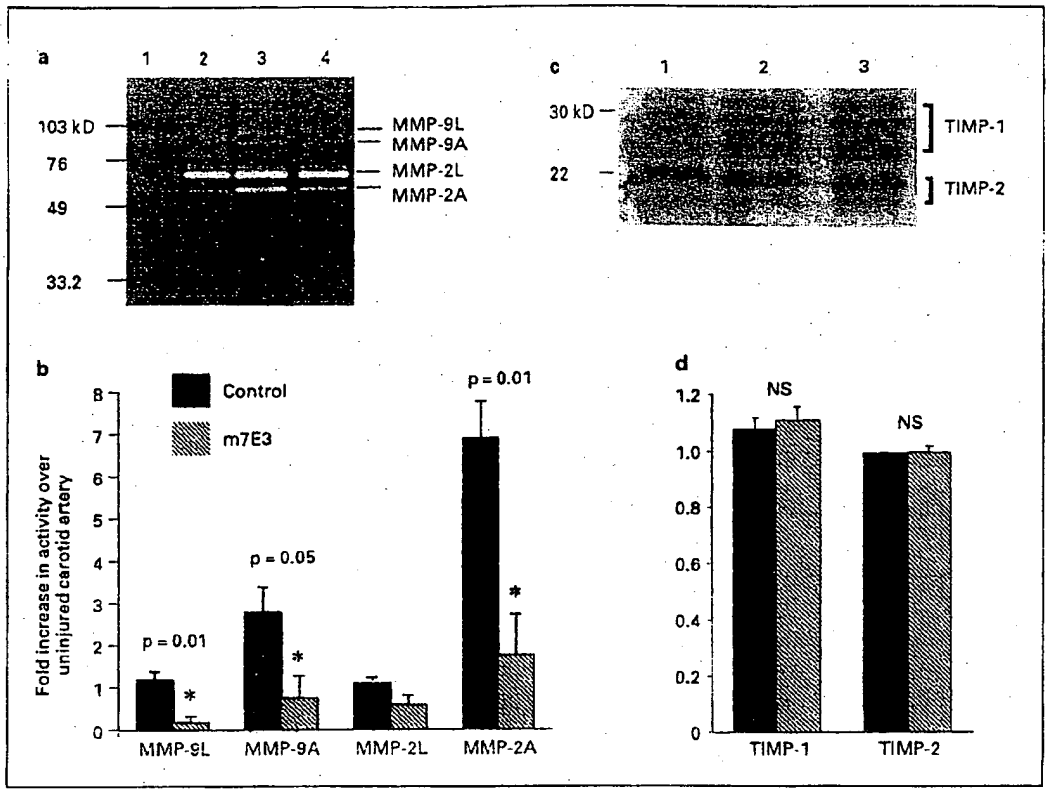

Fig. 2. **a** A representative gelatin zymogram showing MMP activity in rat carotid extracts obtained 4 days after balloon catheter injury. MMP-9 (105 kD latent and 88 kD active) and MMP-2 (72–70 kD latent and 62 kD active) are evident. Lane 1, standards; lane 2, uninjured carotid artery from a saline-treated rat; lane 3, balloon-injured carotid artery from a saline-treated rat; lane 4, balloon-injured carotid artery from an m7E3-treated rat. **b** Results of scanning densitometric analysis of zymograms for MMP activity. Values for each band are expressed as the fold increase over activity of the band in uninjured carotid arteries from saline-treated rats. n = 4 for the control group, and n = 3 for the m7E3-treated group. * indicates significant difference between control and m7E3-treated groups. **c** A representative reverse gelatin zymogram showing activity of TIMP-1 (3 bands from 28 to 31 kD) and TIMP-2 (18 and 21 kD) in rat carotid extracts at 4 days after injury. Lane 1, uninjured carotid artery from saline-treated rat; lane 2, balloon-injured carotid from saline-treated rat; lane 3, balloon-injured carotid from m7E3-treated rat. **d** Results of scanning densitometric analysis of reverse zymograms. Values for each band are expressed as the fold increase over activity of the band in uninjured carotid arteries from saline-treated rats. n = 5 each for the control and m7E3-treated groups. NS = no significant difference between the two groups.

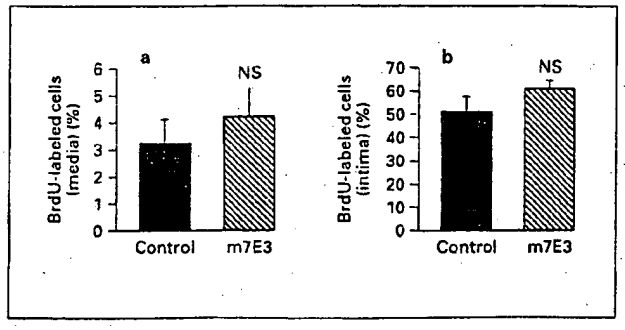

Fig. 3. **a** Medial SMC replication rate 4 days after balloon injury was not significantly different between control and m7E3-treated rats. **b** Intimal SMC replication rate measured at 7 days after injury was not significantly different between control and m7E3-treated rats. Values are mean ± SEM; n = 7 for m7E3-treated rats, and n = 6 for control rats at 4 days. n = 5 for m7E3-treated rats and n = 4 for control rats at 7 days.

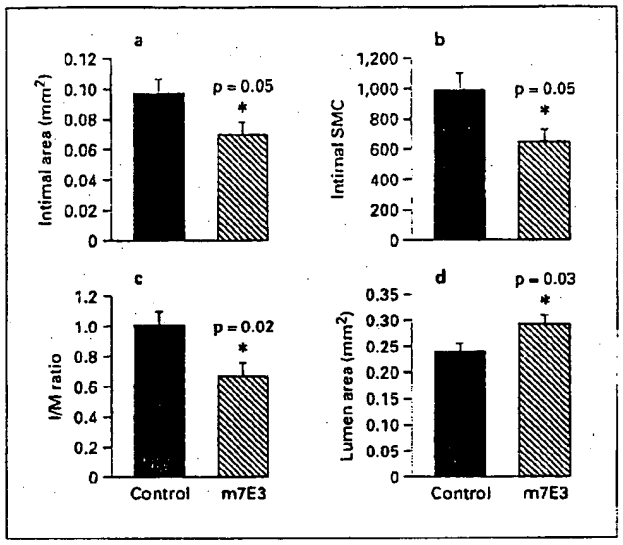

Fig. 4. **a** Intimal cross-section area. **b** Number of SMCs in the intima. **c** Ratio of intimal:medial area. All were significantly decreased in m7E3-treated rats compared to controls at 14 days after carotid injury. **d** Lumen cross-section area was significantly increased in m7E3-treated rats compared to controls. Values are mean ± SEM; n = 8 for m7E3-treated rats, and n = 10 for control rats.

Table 1. Measurement of carotid cross-sections 14 days after balloon injury

	Control (n = 10)	m7E3 (n = 9)	
Medial area, mm²	0.098 ± 0.004	0.107 ± 0.005	NS
Medial SMC number	410 ± 40	423 ± 20	NS
EEL perimeter, mm	2.53 ± 0.09	2.50 ± 0.08	NS

Values are mean ± SEM. EEL = External elastic lamina. NS = m7E3-treated value is not significantly different from control.

Table 2. SMC density 14 days after balloon injury

	Control (n = 5)	m7E3 (n = 4)	
Intimal SMC density, cells/mm²	10,568 ± 652	10,362 ± 624	NS
Medial SMC density, cells/mm²	4,673 ± 489	4,173 ± 62	NS

Values are mean ± SEM. NS = m7E3-treated value is not significantly different from control.

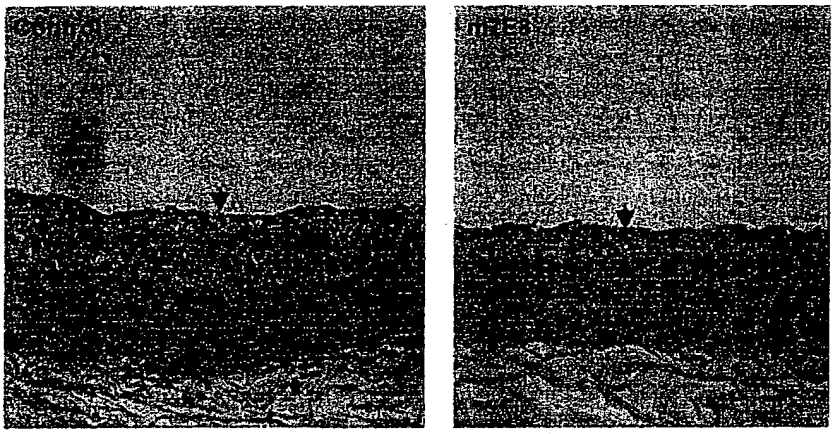

Fig. 5. Carotid cross-sections showing decreased intimal thickening in m7E3-treated rats compared to controls. Arrows indicate the intimal layer. × 400.

nificant 27% reduction in intimal cross-sectional area in m7E3-treated rats vs. control rats at 14 days after injury (fig. 4a, 5). Intimal area in control rats was 0.096 ± 0.009 mm² compared to 0.070 ± 0.008 mm² in m7E3-treated rats (p = 0.05). Intimal SMC number was also decreased in the m7E3-treated rats (p = 0.05) (fig. 4b). By contrast, there were no significant differences in medial cross-sectional area, nor medial SMC number between the control and m7E3 treatment groups (table 1). The ratio of intimal:medial area was significantly decreased by 32% in the m7E3-treated rats (fig. 4c). Lumen area, as measured on carotid cross-sections, was 22% greater in m7E3-treated rats compared to saline controls (fig. 4d). Lumen area measured 0.29 ± 0.02 mm² in m7E3-treated

rats compared to 0.24 ± 0.01 mm^2 in saline controls ($p = 0.03$). The perimeter of the external elastic lamina was measured as an index of vessel remodeling, but there was no significant difference in external elastic lamina perimeter between the treatment and control groups (table 1).

Finally, to assess whether m7E3 treatment affected accumulation of matrix in the injured vessel wall, intimal and medial cell densities were measured. Neither intimal nor medial SMC density differed between the carotids from control and m7E3-treated rats (table 2).

Discussion

In this study we have shown that m7E3, an antagonist of the β3 integrin receptor, inhibited the production of MMPs and dramatically reduced SMC migration into the intima. This inhibition of MMP production and migration resulted in decreased neointimal thickening, and therefore increased lumen area in the carotid artery at 14 days after injury.

Using gelatin zymography, we found that MMP-9 and MMP-2 activities were reduced early after injury in the m7E3-treated rats. The net amount of MMP activity depends on the balance between MMPs and their endogenous inhibitors the TIMPs. Both TIMP-1 and TIMP-2 were expressed in rat carotid arteries, and TIMP-1 activity was slightly increased after injury, but neither TIMP was affected by m7E3. Thus the dominant effect of m7E3 was a reduction in the net activity of the MMPs. Previous work has shown that both MMP-2 and 9 are induced early after arterial injury [19], and are required to facilitate SMC invasion through the medial matrix [20, 21]. Thus in the current study, the decrease in MMP activity in the m7E3-treated rats might explain the decrease in invasion and migration of cells to the intima. MMP-2 and 9 are secreted from cells in latent zymogen form and MMP activation is mediated by the membrane type MMPs [22], the plasminogen activator/plasmin system [23], or in the case of MMP-2, by binding to the αvβ3 receptor [15]. m7E3 had a dramatic inhibitory effect on the active forms of both MMP-2 and MMP-9 (62- and 88-kD bands), suggesting that m7E3 may interfere with the activation process.

We are the first to show reduced MMP activity in vivo following αvβ3 blockade. However, several tissue culture studies suggest that cellular interactions with matrix molecules mediated by integrin receptors can regulate proteinase production. In previous work we showed that osteopontin binding to the αvβ3 integrin receptor stimulated MMP-9 and MMP-1 synthesis [11]. Two other studies have shown that αvβ3 blockade inhibits SMC invasion through matrigel or type I collagen lattices, suggesting that protease activity may have been altered, although proteolytic activity was not directly assessed in these experiments [14, 24]. Taken together, these studies suggest that αvβ3 may be an important receptor stimulating MMP synthesis. This paradigm of αvβ3 signaling for MMPs has been well documented in melanoma cells [25]. Furthermore, it was reported recently that m7E3 blocks tumor angiogenesis, another process that is dependent upon MMP activity [26]. In fact, integrin αvβ3 plays an essential role in angiogenesis by binding and activating MMP-2 on the endothelial cell surface [15]. Taken together, this work indicates that αvβ3 integrins play an important role in regulating MMP synthesis and activity.

Results from the current study confirm and significantly extend our study in which we used another antibody raised against the β3 integrin (F11) [27] and observed decreased SMC migration from media to intima at 4 days after balloon catheter injury of the rat carotid artery [11]. This study also supports work demonstrating a reduction in intimal thickening using other reagents to block the αvβ3 integrin [3], and in addition is more comprehensive in the time course and the breadth of the endpoints which were measured. In the present study we examined the injury response over a 14-day time course, and saw a significant decrease in intimal thickening, intimal SMC number and intima:media ratio with β3 integrin blockade. In our hands, intimal thickening in the injured rat carotid is near maximal at this time point, but we cannot absolutely exclude the possibility of further intimal growth at later times. Our results are in accord with a study showing inhibition of coronary in-stent restenosis following c7E3 treatment in baboons [28]. However, our findings contrast with a study reporting lack of effect of c7E3 post-angioplasty or stenting in monkeys [29]. These contradictory findings may be due to differences between m7E3 and c7E3, differences in dose and administration, or to differences between species.

Our results provide in vivo confirmation of the many in vitro studies which have shown that c7E3 [24,30], and other αvβ3 antagonists reduce SMC migration [4, 6, 8, 31–35]. However, the inhibition of SMC migration is probably not the only mechanism leading to a decrease in intimal lesion formation following m7E3 treatment. m7E3 treatment may have influenced apoptosis, since in vitro studies have shown increased apoptosis of endothelial cells and SMCs following αvβ3 integrin blockade [9, 36, 37]. The amount of intimal thickening can also be influenced by deposition of extracellular matrix. How-

ever, we found no difference in intimal cell density between control and m7E3-treated rats in our studies, leading to the conclusion that at least at this time point, m7E3 treatment did not affect matrix deposition following arterial injury.

We cannot rule out the possibility that the inhibitory effects of m7E3 are mediated through inhibition of platelet or macrophage function, since the antibody binds to platelet αIIbβ3 receptors and macrophage Mac-1 receptors [2, 38]. Fingerle et al. [39] showed that intimal formation was dramatically reduced in rats made thrombocytopenic by injection of antiplatelet antibodies. However, we think it unlikely that m7E3 affected platelet dependent responses in our model. First, we did not observe thrombocytopenia after m7E3 administration. Second, there was no change in platelet deposition after injury. The rat carotid injury model is characterized by deposition of a monolayer of platelets within a few hours after endothelial denudation, and there is no further platelet aggregation and no fibrin deposition [40]. Furthermore, the current data are in accord with previous work showing that 7E3 inhibits platelet aggregation but not other platelet functions, such as adhesion to the vessel wall or matrix [41]. A very recent study reported that PDGF-BB levels in platelets and thromboxane A_2 levels in the plasma were decreased in abciximab (c7E3)-treated rats, resulting in decreased intimal thickening after balloon injury, which the authors postulated was due to decreased thrombus formation [10]. However, these results are difficult to explain, because the dose of antibody used in that study (0.25 mg/kg/day) was likely too low to inhibit rat platelet aggregation. The Fab fragment of 7E3 (abciximab) has a much lower affinity for rat integrins than does the bivalent form used in the current study [16]. In addition the abciximab is cleared much more rapidly in rats than the $F(ab')_2$ version of the antibody used here [16], preventing it from being an effective reagent in rat models. Indeed, a bolus dose of abciximab as high as 20 mg/kg was shown in a previous study to inhibit platelet aggregation for only 10 min [16], suggesting that the almost 100-fold lower dose given over the course of 24 h would provide little to no antiplatelet benefit. The authors did not actually measure thrombosis and a large body of evidence indicates that thrombosis is very rare in the balloon-injured rat carotid. It is unclear why this study produced positive results, but it is unlikely due to a decrease in thrombus formation. Finally, previous studies have shown that very few macrophages infiltrate the injured rat carotid artery [42], so these cells are not likely to play a significant role in this model.

Clinical studies with the murine/human chimeric 7E3 monoclonal antibody fragment (c7E3 Fab, abciximab, ReoPro) show a reduction of in-stent restenosis, ischemic complications, and late mortality after percutaneous intervention [43, 44], particularly in diabetic patients [45]. In contrast, specific anti-αIIbβ3 inhibitors such as eptifibatide and tirofiban, which lack anti-αvβ3 activity, are less effective [1]. These clinical results suggest that the αvβ3 inhibitory effects of c7E3 could be useful in preventing the SMC response to restenosis.

In conclusion, we have shown that administration of m7E3, a β3 integrin inhibitory antibody, was associated with a decrease in SMC MMP activity in the injured rat carotid artery. m7E3 treatment also resulted in a decrease in SMC migration from media to intima, and a significant decrease in neointimal lesion development 2 weeks after injury. These data suggest a role for αvβ3 and a possible mechanism for regulating MMP activity which is a necessary correlate of SMC invasion and migration. In addition, the results of this study may be extrapolated to suggest that αvβ3 blockade could be used to inhibit MMPs and stabilize atherosclerotic plaques, but this will require further investigation in animal models and clinical trials.

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References

1 Bhatt DL, Topol EJ: Current role of platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes. JAMA 2000;284:1549–1558.

2 Tam SH, Sassoli PM, Jordan RE, Nakada MT: Abciximab (ReoPro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and αvβ3 integrins. Circulation 1998;98:1085–1091.

3 Byzova TV, Rabelink TJ, D'Souza SE, Plow EF: Role of integrin αvβ3 in vascular biology. Thromb Haemost 1998;80:726–734.

4 Choi ET, Engel L, Callow AD, Sun S, Trachtenberg J, Santoro S, Ryan US: Inhibition of neointimal hyperplasia by blocking αvβ3 integrin with a small peptide antagonist G*pen*GRGDSPCA. J Vasc Sur 1994;19:125–134.

5 Matsuno H, Stassen JM, Vermylen J, Deckmyn H: Inhibition of integrin function by a cyclic RGD-containing peptide prevents neointima formation. Circulation 1994;90: 2203–2206.

6 Srivatsa SS, Fitzpatrick LA, Tsao PW, Reilly TM, Holmes DF, Schwartz RS, Mousa SA: Selective αvβ3 integrin blockade potently limits neointimal hyperplasia and lumen stenosis following deep coronary arterial stent injury: Evidence for the functional importance of integrin αvβ3 and osteopontin expression during neointima formation. Cardiovasc Res 1997;36: 408–428.

7 Coleman KR, Braden GA, Willingham MC, Sane DC: Vitaxin, a humanized monoclonal antibody to the vitronectin receptor (αvβ3), reduces neointimal hyperplasia and total vessel area after balloon injury in hypercholesterolemic rabbits. Circ Res 1999;84:1268–1276.

8 Slepian MJ, Massia SP, Dehdashti B, Fritz A, Whitesell L: β3-integrins rather than β1-integrins dominate integrin-matrix interactions involved in postinjury smooth muscle cell migration. Circulation 1998;97:1818–1827.

9 van der Zee R, Murohara T, Passeri J, Kearney M, Cheresh DA, Isner JM: Reduced intimal thickening following αvβ3 blockade is associated with smooth muscle cell apoptosis. Cell Adhes Commun 1998;6:371–379.

10 Wu CH, Chen YC, Hsiao G, Lin CH, Liu CM, Sheu JR: Mechanisms involved in the inhibition of neointimal hyperplasia by abciximab in a rat model of balloon angioplasty. Thromb Res 2001;101:127–138.

11 Bendeck MP, Irvin C, Reidy MA, Smith LA, Mulholland D, Horton MA, Giachelli CM: Smooth muscle cell matrix metalloproteinase production is stimulated via αvβ3 integrin. Arterioscler Thromb Vasc Biol 2000;20:1467–1472.

12 Bishop GG, McPherson JA, Sanders JM, Hesselbacher SE, Feldman MJ, McNamara CA, Gimple LW, Powers ER, Mousa SA, Sarembock IJ: Selective αvβ3-receptor blockade reduces macrophage infiltration and restenosis after balloon angioplasty in the atherosclerotic rabbit. Circulation 2001;103:1906–1911.

13 Dollery CM, McEwan JR, Henney AM: Matrix metalloproteinases and cardiovascular disease. Circ Res 1995;77:863–868.

14 Kandu S, Kuzuya M, Ramos MA, Koike T, Yoshino K, Ikeda S, Iguchi A: Matrix metalloproteinase and αvβ3 integrin-dependent vascular smooth muscle cell invasion through a type I collagen lattice. Arterioscler Thromb Vasc Biol 2000;20:998–1005.

15 Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvβ3. Cell 1996;85:683–693.

16 Sassoli PM, Emmell EL, Tam SH, Trikha M, Zhou Z, Jordan RE, Nakada MT: 7E3 F(ab')2, an effective antagonist of rat αIIbβ3 and αvβ3, blocks in vivo thrombus formation and in vitro angiogenesis. Thromb Haemost 2001;85:896–902.

17 Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Lab Invest 1983;49:327–333.

18 Hou G, Vogel W, Bendeck MP: The discoidin domain-receptor tyrosine kinase DDR1 in arterial wound repair. J Clin Invest 2001;107: 727–735.

19 Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA: Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. Circ Res 1994;75: 539–545.

20 Bendeck MP, Irvin C, Reidy MA: Inhibition of matrix metalloproteinase activity inhibits smooth muscle cell migration but not neointimal thickening after arterial injury. Circ Res 1996;78:38–43.

21 Zempo N, Koyama N, Kenagy RD, Lea HJ, Clowes A: Regulation of vascular smooth muscle cell migration and proliferation in vitro and in injured rat arteries by a synthetic matrix metalloproteinase inhibitor. Arterioscler Thromb Vasc Biol 1996;16:28–33.

22 Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI: Mechanism of cell surface activation of 72-kD type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. J Biol Chem 1995;270:5331–5338.

23 Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D: Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet 1997;17:439–444.

24 Blindt R, Bosserhoff AK, Zeiffer U, Krott N, Hanrath P, vom DJ: Abciximab inhibits the migration and invasion potential of human coronary artery smooth muscle cells. J Mol Cell Cardiol 2000;32:2195–2206.

25 Seftor REB, Seftor EA, Stetler-Stevenson WG, Hendrix MJC: The 72 kD type IV collagenase is modulated via differential expression of αvβ3 and α5β1 integrins during human melanoma cell invasion. Cancer Res 1993;53:3411–3415.

26 Varner JA, Nakada MT, Jordan RE, Coller BS: Inhibition of angiogenesis and tumour growth by murine 7E3, the parent antibody of c7E3 Fab (abciximab; ReoPro). Angiogenesis 1999; 3:53–60.

27 Helfrich MH, Nesbitt SA, Horton MA: Integrins on rat osteoclasts: characterization of two monoclonal antibodies (F4 and F11) to rat β3. J Bone Min Res 1992;7:345–351.

28 Marijianowski MM, Nakada MT, Jordan RE, Jakubowski JA, Sundell BI, Kelley AB, Chronos NF, Hanson SR: Abciximab reduces stent restenosis in non-human primates. Circulation 1999;100:I-697.

29 Deitch JS, Williams JK, Adams MR, Fly CA, Herrington DM, Jordan RE, Nakada MT, Jakubowski JA, Geary RL: Effects of β3-integrin blockade (c7E3) on the response to angioplasty and intra-arterial stenting in atherosclerotic nonhuman primates. Arterioscler Thromb Vasc Biol 1998;18:1730–1737.

30 Baron JH, Moiseeva EP, De Bono DP, Abrams KR, Gershlick AH: Inhibition of vascular smooth muscle cell adhesion and migration by c7E3 fab (abciximab): A possible mechanism for influencing restenosis. Cardiovasc Res 2000;48:464–472.

31 Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM: Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circ Res 1994; 74:214–224.

32 Yue T-L, Mckenna PJ, Ohlstein EH, Farach-Carson MC, Butler WT, Johanson K, McDevitt P, Feuerstein GZ, Stadel JM: Osteopontin-stimulated vascular smooth muscle cell migration is mediated by β3 integrin. Exp Cell Res 1994;214:459–464.

33 Jones JI, Prevette T, Gockerman A, Clemmons DR: Ligand occupancy of the αvβ3 integrin is necessary for smooth muscle cells to migrate in response to insulin-like growth factor I. Proc Natl Acad Sci USA 1996;93:2482–2487.

34 Bilato C, Curto KA, Monticone RE, Pauly RR, White AJ, Crow MT: The inhibition of vascular smooth muscle cell migration by peptide and antibody antagonists of the alphavbeta3 integrin complex is reversed by activated calcium/calmod. J Clin Invest 1997;100:693–704.

35 Itoh H, Nelson PR, Murcebe L, Horowitz A, Kent KC: The role of integrins in saphenous vein vascular smooth muscle cell migration. J Vasc Sur 1997;25:1061–1069.

36 Brooks PC, Montgomery AMP, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA: Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 1994;79:1157–1164.

37 Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia R, Giachelli CM: NF-κB mediates αvβ 3 integrin-induced endothelial cell survival. J Cell Biol 1998;141:1083–1093.
38 Simon DI, Xu H, Ortlepp S, Rogers C, Rao NK: 7E3 monoclonal antibody directed against the platelet glycoprotein IIb/IIIa cross-reacts with the leukocyte integrin Mac-1 and blocks adhesion to fibrinogen and ICAM-1. Arterioscler Thromb Vasc Biol 1997;17:528–535.
39 Fingerle J, Johnson R, Clowes AW, Majesky MW, Reidy MA: Role of platelets in smooth muscle cell proliferation and migration after vascular injury in rat carotid artery. Proc Natl Acad Sci USA 1989;86:8412–8416.
40 Reidy MA, Yoshida K, Harker LA, Schwartz SM: Vascular injury: Quantification of experimental focal endothelial denudation in rats using indium-111-labeled platelets. Arteriosclerosis 1986;6:305–311.
41 Jordan RE, Wagner CL, Mascelli M, Treacy G, Nedelman MA, Woody JN, Weisman HF, Coller BS: Preclinical development of c7E3 Fab; a mouse/human chimeric monoclonal antibody fragment that inhibits platelet function by blockade of GPIIb/IIIa receptors with observations on the immunogenicity of c7E3 Fab in humans; in Horton MA (ed): Adhesion Receptors as Therapeutic Targets. Boca Raton, CRC Press, 1996, pp 281–305.
42 Ferns GA, Reidy MA, Ross R: Balloon catheter deendothelialization of the nude rat carotid. Response to injury in the absence of functional T-lymphocytes. Am J Pathol 1991;138:1045–1057.
43 Topol EJ, Ferguson JJ, Weisman HF, Tcheng JE, Ellis SG, Kleiman NS, Ivanhoe RJ, Wang AL, Miller DP, Anderson KM, Califf RM: Long-term protection from myocardial ischemic events in a randomized trial of brief integrin β3 blockade with percutaneous coronary intervention. JAMA 1997;278:479–484.
44 The Epilog Investigators: Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. N Engl J Med 1997;336:1689–1696.
45 Lincoff AM, Califf RM, Moliterno DJ, Ellis SG, Ducas J, Kramer JH, Kleiman NS, Cohen EA, Booth JE, Sapp SK, Cabot CF, Topol EJ: Complementary clinical benefits of coronary-artery stenting and blockade of platelet glycoprotein IIb/IIIa receptors. N Engl J Med 1999; 341:319–327.

Management of restenosis after coronary intervention

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It has been almost 20 years since the first report of percutaneous transluminal coronary angioplasty (PTCA) by Gruentzig[1] in 1977. Currently interventional cardiology is widely applied and uses a broad range of techniques. However, despite the sophistication of the new techniques, recurrence of coronary arterial stenoses requiring repeated procedures has persisted.

DEFINITION

Restenosis is the decrease of the vessel lumen at the site of the procedure, occurring in 40% to 50% of cases. Restenosis usually presents with recurrent chest pain, but it may also be asymptomatic. The majority of restenoses develops within the first 3 to 4 months after the procedure; lumen narrowing is usually completed by 6 months.[2, 3] Restenosis can be defined as histologic, clinical, or angiographic. Angiographically significant restenoses are those with >50% stenosis or with >30% lumen reduction from the initial successful result.[4, 5] Serruys et al.[4] defined the abnormal restenotic process as lumen narrowing that exceeds the mean by two standard deviations or 0.72 mm (with 2.5% false positives) and showed that "the more you gain, the more you lose." Kuntz et al.[6] identified that minimal lumen diameter (MLD) achieved with the intervention is the most important parameter for restenosis regardless of the specific technique, implying that "bigger is better."

PATHOGENESIS

The sequence of events that leads to restenosis begins with the arterial physical injury created by the balloon or other devices. Thereafter a complex cascade begins, involving inflammatory and thrombotic mechanisms, intimal hyperplasia, and vessel recoil. The restenotic process has been described as similar to wound healing. A classification of the pathogenesis of restenosis into phases may apply on the basis of recent data: early elastic recoil (first day), formation and organization of mural thrombus (first 2 weeks), and neointimal proliferation (first 3 months). Chronic geometric changes of the vessel occur at the same time.

Early elastic recoil. The arterial stretch caused by balloon dilation is acutely followed by lumen loss. The mechanism has been attributed to elastic recoil, which occurs within hours of the revascularization and may be up to 50% immediately after the procedure. An early (24 hour) post-procedure angiogram has been used to predict future restenosis[7] with a rate of 73.6% for the lesions that had lumen loss >10% (<100%) and only 9.8% for lesions that diminished by <10%. Early recoil may possibly have a significant role in late restenosis, probably when the vessel has not been severely injured and the lesion mainly consists of smooth muscle cells. When arterial injury is severe, mural thrombus forms acutely.[8]

Formation of mural thrombus. The technical variables that correlate with successful initial angiographic result (balloon size, inflation pressure, and time) cause deep arterial injury, resulting in a tear through the intima, vessel stretch, and plaque compression. Endothelial denudation deprives the vessel wall from endothelium-derived antithrombotic factors (e.g., endothelium-derived relaxing factor, prostaglandin I_2, plasminogen activator inhibitor, tissue-type plasminogen activator) and allows platelet adhesion and aggregation with exposure to subendothelial matrix and collagen. Platelets bind to cell adhesion molecules and subsequently degranulate. The final pathway of platelet aggregation involves the binding of the IIb/IIIa integrin to fibrinogen, von Willebrand factor, and fibronectin.

Sequential development and lysis of platelet thrombus with fluctuation of blood flow has been associated with the development of neointimal hyperplasia in the dog model. Locally decreased blood flow and shear stress augment this process. The larger the postprocedure stenosis, the greater the flow fluctuation leading to increased platelet adhesion and intimal hyperplasia (hence the importance of ostial, angulated, and tortuous lesions). Therefore a hemo-

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dynamically compromised initial outcome may trigger an unfavorable response as opposed to a minimal residual stenosis.[8-10]

Platelets degranulate, releasing a large number of procoagulant, vasoconstrictive, and mitogenic substances—including thrombin, platelet-derived growth factor (PDGF), thromboxane A2, serotonin, von Willebrand factor, adenosine diphosphate (ADP), fibronectin, factor V, and fibrinogen—leading to formation of deep intramural thrombus[11] inaccessible to the action of heparin (clot-bound and matrix-bound thrombin).[12, 13] The varying consistency of de novo atherosclerotic plaques reflects the composition of the surface exposed after PTCA. Thrombogenicity is highest on top of plaques with exposed lipid-rich core but also very high at the residual mural thrombus itself.[8]

Mural thrombi appear to function as a substrate for smooth muscle cell (SMC) migration and proliferation. This function may be mediated specifically by thrombin, platelets, monocytes, macrophages, and neutrophils that are also attracted locally. A recent report localized the macrophage concentration at the site of thrombus formation and plaque rupture in unstable as opposed to stable lesions.[14] Mural thrombus organization involves both SMC proliferation and extracellular matrix (ECM) synthesis and is probably related to all the previous factors, which are also involved in the third phase of the restenotic process.

Neointimal proliferation. This phase is predominantly characterized by SMC proliferation and ECM synthesis at the site of the vascular injury, thereby causing lumen narrowing. In the *first part* of this phase, SMC activates in association with mural thrombosis and growth factors such as fibroblast growth factor, which is the most potent stimulant of SMC proliferation. PDGF and thrombin locally accumulate in response to the ongoing thrombotic process. The multiple effects of thrombin on the vessel wall may lead to the incorporation of the mural thrombus by connective tissue. Thrombin is a chemoattractant for monocytes and other inflammatory cells. Although thrombin receptor activity is confined to endothelium in a normal arterial wall, it is expressed in SMC and macrophage areas of atherosclerotic lesions.[8] Thrombin may therefore be involved in plaque inflammation and fibrosis leading to mural thrombus organization.

During the *second part* of this phase, PDGF (also expressed by macrophages and SMC) has been shown to induce SMC migration from the media to the intima, where the cells appear phenotypically different and proliferate under the action of basic fibroblast growth factor (βFGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor β (TGF-β), and other growth factors and cytokines.[15, 16] Data obtained from multiple animal models have established the pivotal role of PDGF-BB (platelet-secreted) for SMC migration.[17]

The *third part* of this phase involves intimal SMC proliferation, which decreases after 2 to 4 weeks. Any further luminal narrowing is from ECM produced. Whether the end of SMC proliferation is signaled directly by their contact with the excess of ECM formed or by the regrowth of normal endothelium has yet to be determined. Reendothelialization can achieve nitric oxide–mediated SMC inhibition and cessation of the ongoing vessel wall exposure to platelets and PDGF-BB.[8]

ECM is formed by SMC of the proliferative-synthetic phenotype. Probably under the effect of TGF-β, the ECM of the restenotic fibrocellular tissue differs from the atherosclerotic tissue, with preferential expression of the proteoglycan biglycan and collagen types I and III in rather variable density and random alignment.[18] Proteoglycan production increases the volume of the neointima, and the addition of collagen may contribute to late vessel shrinkage at the site of the lesion analogous to the final wound retraction. Collagen and matrix formation are thought to be completed by the fourth month after the initial event,[3, 8, 19] probably because of the contact with SMC.

Geometric arterial changes. The vessel wall undergoes geometric changes in response to injury. In atherosclerotic vessels a chronic focal enlargement of the artery occurs in response to the plaque increase to preserve blood flow.[20, 21] In restenosis, the initial stretch leads to destruction of vasa vasorum (particularly abundant around the atherosclerotic areas), possible hypoxia of the vessel wall, and compression of the media, resulting in SMC injury and increased DNA synthesis.

Intravascular ultrasound (IVUS) has become an important means to understanding the concept of restenosis. IVUS imaging after PTCA has shown that there is more axial plaque redistribution than compression and that failure to cause any dissection leads to early lumen loss by elastic recoil (15% of cases). Mintz et al.[22] described that restenotic lesions lead to contraction of the artery, contributing to 66% of the late lumen narrowing. The same investigators also reported the residual plaque burden after the intervention to be the most powerful predictor of restenosis.[23]

Three types of lumen progression after the proce-

dure have been described: late lumen gain (with increased external elastic membrane [EEM] diameter and smaller degree of intimal hyperplasia), late lumen loss (with minimal change in EEM diameter and significant intimal hyperplasia but still >50% patency), and restenosis (decrease in EEM diameter, increased intimal hyperplasia with <50% patency). These changes of the EEM dimensions may be caused by local hemodynamic characteristics after the intervention and the destruction of the architecture of the vessel wall.

PREDICTION OF RESTENOSIS

Identifying the lesions that will result in angiographically significant restenosis may lead to an understanding of its pathogenesis.[24, 25] The only clinical characteristics that increase the likelihood of restenosis are unstable angina (crescendo or recent onset) and diabetes mellitus (especially insulin-dependent). In unstable angina lesions, the culprit vessel shows more late lumen loss than the nonculprit.[26] Smoking has not been definitively associated with restenosis, and contradictory results have been found with hyperlipidemia. Recent reports have found an association with high Lp(a) levels.[27] Hypertension, end-stage renal disease, and vasospastic angina may predispose to restenosis.

The M-HEART study used a systematic approach to identify anatomic and procedural factors associated with low, intermediate, and high incidence of restenosis.[28] Native coronary artery diameter and lesion length, left anterior descending or saphenous vein graft lesions and increased percentage of stenosis pre- and postprocedure correlated best with restenosis. The low-risk group had a restenosis rate of 20%, whereas restenosis in the high-risk group was 61% (overall restenosis rate was 41%).

Other studies have reported a higher incidence of restenosis for lesions located proximally, at a bifurcation, or a bend. Unstable angina patients with complex lesions, intraluminal thrombosis, and reduced Thrombolysis in Myocardial Infarction trial flow have higher restenosis rates than lesions without evidence of thrombus.[24] Another subset of patients with high restenosis rates are those with totally occluded vessels. In a recent review of 2950 patients with 3583 lesions and 266 totally occluded vessels, the restenosis rate was 45% for the totally occluded lesions (19% as recurrent total occlusions) and 34% for the other stenoses (5% as recurrent total occlusions).[29] The variables associated with restenosis were the balloon inflation time and the presence of thrombus.

NEWER REVASCULARIZATION DEVICES

The high incidence of restenosis with balloon angioplasty (40%) and its inadequacy for technically demanding lesions led to the development of newer interventional devices. Although they all have a more sophisticated approach to lesions, significant impact on the postprocedure restenosis rate has not been made except with the use of intracoronary stents.[6]

In 1964 Dotter and Judkins first suggested the use of an endovascular stent, and the first intracoronary stent was placed in 1987 by Sigwart.[1] Their initial use only for "bailout" intervention expanded after positive results of recent trials. The BENESTENT[30] trial in patients with chronic stable angina and a single lesion showed an improved 6-month restenosis rate (22% vs 32%) and decreased need for repeat revascularization (10% vs 20%), at the expense of increased peripheral vascular complications (14% vs 3%) because of the need for more intense anticoagulation in the stent group compared with the PTCA group. Similar results were obtained in the STRESS trial in patients with symptomatic coronary artery disease.[31]

Conforming to the rule "bigger is better," the MLD achieved with stents was larger than in the PTCA group and remained so at follow-up despite the greater late loss observed. The immediate postprocedure elastic recoil is greatly reduced with stents because of their rigid structure.

Stents also cause some focal medial necrosis at the site of the indentation and mainly offer a geometrical support (scaffolding) to keep the vessel open. This function has been studied with IVUS, which is used routinely after stent placement for proper expansion and deployment.[32] Apart from diminishing acute elastic recoil, stents eliminate chronic geometric arterial changes (shrinking). Stent restenosis seems primarily caused by neointimal hyperplasia, which appears to be more pronounced in stent cases than other interventions. Besides the mechanical wall support, stents modify the restenotic process by providing markedly increased blood flow to the treated vessel.[33]

IVUS application for optimal stent deployment, especially against eccentric plaques, ensures the elimination of any thrombogenic interspace (low flow) between stent and vessel wall and may obviate the need for anticoagulation and decrease associate vascular complications but also affect the long-term restenosis by allowing faster reendothelialization.[23, 33] This potential contribution of the postintervention IVUS result to restenosis is being evaluated in large trials.

ADJUVANT MEDICAL THERAPY FOR PREVENTION OF RESTENOSIS

Systemic pharmacologic therapy. Multiple agents have been tried, with mostly discouraging results. The various classes of drugs evaluated is summarized along with their potential mechanism against the pathogenesis of restenosis (Table I).

Antithrombotic agents. Because the initial event after injury is platelet-thrombus formation over the denuded endothelium, expecting improvement with antiplatelet agents is reasonable. Dipyridamole, ticlopidine, and thromboxane A2 inhibitor studies have had negative results, and only the meta-analysis of all the various aspirin trials was able to show a trend toward decreased restenosis, substantiating the current wide use of this agent.[25] The prostacyclin-analog ciprostene, however, has shown some initial positive results.[34]

The development of a murine antibody against platelet IIb/IIIa integrin has been a novel approach to pharmacotherapy. After genetic engineering (because of immunogenic concerns) to a chimeric form, this antibody was used during PTCA and showed 80% inhibition of platelet aggregation. In the EPIC trial, involving 2099 patients undergoing high-risk PTCA with primary end point being 30-day major coronary event rate, the chimeric monoclonal antibody was administered as an intravenous bolus at least 10 minutes before the procedure, with or without a 12-hour infusion, in addition to aspirin and heparin therapy.[35] This trial showed significant reduction in primary end points (13% placebo group, 8% bolus plus infusion group), but increased bleeding complications attributed to heparin dosage.[36] The 6-month follow-up of this population showed decreased rates of target vessel revascularization (17% vs 22%) and major ischemic events (27% vs 35%) for the bolus plus infusion group compared with placebo, respectively.[37] In all comparisons the group treated with only drug bolus had intermediate results. The potential mechanism of this antibody is the "passivation" of the cell surface at the time of the injury. Other IIb/IIIa blockers (integrelin, MK-383) are currently being evaluated.

Heparin inhibits platelet function and SMC proliferation in addition to thrombin inactivation when bound to the cofactor antithrombin-III (AT-III). However, heparin has failed to show beneficial effects on restenosis,[38] possibly because of its inability to bind thrombin situated deeply in the subendothelium (supposedly important for SMC proliferation as well). The first drug to obviate the need for heparin during PTCA was the synthetic antithrombin bivalirudin (Hirulog) in 1993, which is currently tested for possible benefit on restenosis. Naturally occurring hirudin is more effective than heparin in inhibiting mural thrombus, probably because of its smaller size and the fact that it can function without a cofactor.[39] Hirudin use during PTCA showed a trend toward fewer peri-intervention ischemic events; however, the HELVETICA trial failed to demonstrate a benefit on restenosis.[40]

Low molecular weight heparin preparations (e.g., enoxaparin) are fragments of the usual heparin with slower clearance and increased activity for factor Xa. Despite encouraging animal data, these agents failed to show restenosis benefit in humans, possibly because of the lower dose used.[41] A synthetic tripeptide (structure similar to fibrinopeptide A) has been delivered locally with the hydrogel balloon and inhibited platelet-dependent thrombus in the pig model.[42] A 15-nucleotide (ss-DNA) that has a specific direct thrombin-inhibitory effect is also being experimentally evaluated.[43]

Lipid-lowering drugs. Intensive treatment with a cholesterol-lowering agent (lovastatin) after PTCA failed to show benefit on restenosis despite aggressive LDL-cholesterol lowering achieved.[44] Treatment with various doses of omega-3 fatty acids (fish oil) has been efficacious in multiple small trials, and a meta-analysis showed significant reduction of clinical and angiographic end points for restenosis.[25] Larger studies are underway. These agents have in-

Table I. Therapeutic aims against pathogenesis of restenosis

Response to vessel injury	*Targeted therapy*
Early elastic recoil	Achievement of greater MLD Stents
Mural thrombus formation	Antithrombotic agents Antiplatelet agents Vessel wall passivation Rapid re-endothelialization Molecular therapy Strict control of diabetes (?)
Neointimal proliferation SMC Activation SMC Migration SMC Proliferation/ ECM formation	Antiproliferative agents Molecular therapy Rapid re-endothelialization Vessel wall passivation Omega-3 fatty acids (?) Local irradiation/radioactivity (?) Local ACE-inhibitors (?) Strict control of diabetes (?)
Chronic geometric changes	Stents Achievement of greater MLD (?)

Table II. Local drug delivery systems

Iontophoretic porous balloon
Balloon with hydrophilic polyacrylic polymer (hydrogel)
Infusion sheath
Double balloon system
Wolinsky's perforated balloon
Channel catheter
Transport porous catheter
Dispatch catheter
Rheolytic system
Microinjector with retractable needles
Balloon over a stent
Ultrasonic energy and radiofrequency (local therapy)
Stent-based applications
Biodegradable drug releasing polymer stents
Dacron stents
Silicone stents
Polyether urethane urea stents
High molecular weight polylactic acid stents
Removable stents (nitinol) with drug eluting polyurethane coating
Fibrin-coated metallic stents
Stents with genetically modified endothelial cells
Stents with radioactive substance

hibited intimal hyperplasia in animals, possibly because of serum lipid reduction and decreased platelet aggregation.

Antiproliferative agents. Agents that inhibit cell migration and proliferation have shown some effect in preventing restenosis. Trapidil inhibits thromboxane-A2 and a competitive PDGF receptor antagonist. The STARC trial involved 354 patients assigned to 100 mg three times daily of trapidil or aspirin begun at least 3 days before PTCA and continued for 6 months.[45] This trial showed reduction in the restenosis rate for the trapidil group (26% vs 44%, or 31% vs 45%, according to the definition applied), which was statistically significant. The trapidil group had less unstable angina (18% vs 31%) and was also more angina-free (74% vs 56%) at 6 months.

Angiopeptin is a somatostatin analog (cyclic octapeptide) that inhibits IGF-1 and fibroblast growth factor; it has been shown to limit myointimal thickening and restore vasodilatory response of the vessel to acetylcholine. This agent has been shown to lower the clinical event rate (mainly repeat revascularization) at 12-month follow-up after PTCA in a randomized double-blind placebo control trial of 553 patients (28% vs 36%) but without significant impact on angiographic restenosis.[46]

Vasodilators. Vasodilating agents have been tried without supportive data. Favorable trend toward restenosis prevention by calcium-channel blockers is only evident in pooled data.[26] Ketanserin, a serotonin receptor antagonist (inhibits vasoconstriction, platelet activation, and the mitogenic effect of serotonin on SMC) failed to demonstrate positive clinical or angiographic outcome 6 months after PTCA[47] in a randomized trial. The angiotensin-converting enzyme inhibitors have been investigated for possible restenosis benefit. Although data in the rat model were promising, clinical trials had negative results. However, the doses used were significantly lower than those needed to produce favorable results in the animal models, and drug administration was initiated after PTCA.[48]

Local drug delivery. New delivery systems have been developed in an attempt to reduce restenosis by directly administering pharmacologic agents to the lesion that either cannot be delivered by other means or need higher local concentration, which would be impossible to achieve with systemic administration because of adverse side effects. Stents can be coated with various substances or genetically engineered endothelial cells or processed to locally deliver radioactivity or radiation. Heparin-coated stents without postprocedure anticoagulation have been reported to reduce subacute thrombosis compared with noncoated stent placement after systemic anticoagulation.[49] Their effect on restenosis is currently being evaluated in the BENESTENT II trial. Many transcatheter delivery systems have also been developed (Table II).

Evaluation of all these new systems and devices is difficult because their failure may be caused by other factors, including the model selected and the intricate details of their technical characteristics. Arterial size, prior injury, plaque thickness, and extent of disease differ in all experiments. Apposition of the delivery system to the vessel wall may cause damage but at the same time help eliminate anatomic barriers and increase the depth of drug diffusion. Human atherosclerotic plaques appear nonpermeable, but their abundant vasa vasorum may facilitate local diffusion.[42, 50]

Pharmacologic agents. Some agents failed to decrease restenosis or had equivocal outcome in clinical trials despite promising results in animal models. Their failure in human beings was partly attributed to relative underdosage to avoid side effects. Drugs such as the angiotensin-converting enzyme inhibitors are now being tried in this field. Neointimal hyperplasia appears to be the most specific target of local drug delivery because of the proximity of the event to the arterial lumen and therefore the catheter used. The knowledge of how deep in the wall each drug needs to be delivered is essential at this point.

Some authors have suggested that it is essential to achieve adventitial saturation for adequate impact on restenosis.[51]

The use of stents as delivery systems closely correlates with their successful use as interventional methods. Their main advantage would be the ability to integrate local drug delivery with the revascularization device. An additional factor that makes them desirable for local delivery is that stent restenosis can be mainly attributed to neointimal hyperplasia through the metallic mesh, thereby isolating a specific target for local therapy. Antithrombotic and antiproliferative medications could be tried for local delivery, either with catheter systems or with stents. In attempt to achieve optimal combination of safety and maximal efficacy every medication might be tried for both systemic and local delivery. However, reliable evaluation of the drugs themselves should follow validation of the specific delivery system used.

Molecular therapies. With the growing understanding of the pathophysiologic characteristics of restenosis, investigators have tried to control and transform it at the molecular level. Targets for molecular therapy can involve all pathways of the restenosis process. Endothelial cells, for example, can be engineered to express inhibitors (e.g., tissue-type plasminogen activator). Platelet adhesion targets could be the integrins Ia-collagen and Ib-IX, and platelet aggregation can be inhibited by blocking the IIb/IIIa integrin. Intimal hyperplasia can be attenuated by antibodies to PDGF and βFGF.[17] Antisense oligos target SMC proliferation, which is important for restenosis and occurs over a limited amount of time. Oncogenes and cyclins have been reported to be successfully inhibited. Extracellular matrix formation is a late phenomenon, and agents such as interferon-α, interferon-γ, and HMG CoA (3-hydroxy 3-methylglutaryl coenzyme A) reductase inhibitors, or antioxidants could reduce it.[52]

Lipofection is the achievement of DNA delivery to the target cell using cationic liposomes, which are positively charged artificial lipid vesicles with negatively charged DNA inside. The rate of transfer depends on cell type and is facilitated by endothelial denudation and cell proliferation (high for restenotic SMC). The liposomes enter the cell by receptor-mediated endocytosis. Reagents that inhibit lysosomal degradation increase the transfection rate.

Viral vectors can be used for transfection. Retroviruses lead to DNA integration in the chromosome and thereby increase the potential for insertional mutagenesis. In vivo transfection needs cell replication and is inhibited by serum and complement. Adenoviral vectors enter the cell with receptor-mediated endocytosis and escape lysosomal degradation because of their capsid proteins. However, they have limited expression (3 weeks), probably because of cell death, cause inflammation, and have extensive infecting potential of various other cell types (brain, liver). Their unique property of avoiding lysosomal degradation has been used in the formation of a transfecting complex, where the DNA is bound to inactivated adenovirus, which in turn is coupled to a ligand-like transferrin and allows the complex to be identified by the ligand receptor. The hemaglutination virus of Japan has also been used for transfection.[52]

Indirect gene transfer has been attempted with removal of endothelial cells, ex vivo genetic engineering (e.g., for TPA expression), and reimplantation either with stents or with balloon devices. Polymer gels with the hydrogel balloon or with direct periadventitial apposition have been used.[50]

Gene therapy includes two possible approaches. The gene transferred either encodes a protein that will be retained in the cell or one that will be secreted. Thus the desired effect is exerted in more cells than those actually transfected. Adenoviral vectors have been used for transfection of SMC with the gene encoding herpes simplex virus thymidine kinase followed by gancyclovir administration,[53] which selectively kills the herpes simplex virus thymidine kinase expressing cells and neighboring cells (innocent bystander effect). Insertion of a constitutively active negative regulator (retinoblastoma gene product) with an inactivated adenoviral vector has resulted in cell stasis.[54]

Antisense oligonucleotides are short segments of DNA that bind the messenger ribonucleic acid, leading to inhibition of translation. An important limitation is the possibility for nonselective binding of other cellular proteins. Proto-oncogenes and cell cycle–regulating genes have been successfully inhibited in animal models, but negative results exist as well.[55, 56] Aptamers are short DNA or ribonucleic acid strands that can bind specific proteins and successfully inhibit thrombin activity in vitro.[43]

The "Trojan horse" approach takes advantage of the expression of large number of cell surface receptors for growth factors by the proliferating SMC. Recombinant fusion proteins made of a potent toxin and a peptide ligand-to-cell-surface receptor (growth factor) are available. The possible candidate for the toxins include *Pseudomonas* exotoxin, *E. coli* exotoxin, diphtheria toxin, and cholera toxin. The candidate for the growth factors can be EGF, TGFa, βFGF, and

interleukin-2. The complex EGF-diphtheria toxin has been administered in human atherosclerotic plaque outgrowth leading to inhibition of proliferation.[57]

Inactive substances are also systemically delivered and locally activated. These substances are the photodynamic approaches to restenosis[58] with light-excitable photosensitizes such as the hematoporphyrin derivatives and cyanide products. Psoralen and psoralens and ultraviolet A lead to 28-day stasis of cell proliferation without necrosis after single exposure. Very low dose ultraviolet or radiation delivered locally have been used alone. The reservations about these agents are the potential for the necrosed cells to produce mitogens with second wave of cell proliferation and their cutaneous photosensitivity (dose limiting).

Conclusions. The high incidence of restenosis after coronary intervention has been disappointing. However, one should note the discrepancy between immediate and early postintervention angiographic results. In all restenosis trials, baseline MLD is considered the immediate postangioplasty one. Given that significant early lumen loss may occur within even hours after the procedure, restenosis rates may possibly have been systematically overestimated in all trials. Conceivably, early rather than late lumen loss may be a more significant determinant of the ultimate lumen narrowing. Recent encouraging data from early platelet inhibition and antiproliferative therapy offer a new window of opportunity for a breakthrough in pharmacotherapy for preventing restenosis. For the first time an interventional device (stents) has proven efficacious in late patency, leading to a new insight in restenosis pathogenesis. Preprocedural clinical characteristics and intravascular ultrasound imaging may better define predictors of restenosis. Several unresolved issues exist in the molecular aspects of restenosis prevention. The exact implications of the intimal and medial necrosis are not defined, and whether how much of this action may actually be too much is unclear. Because we are actually inhibiting the normal protective vessel reaction of healing after injury, the effect could potentially be deleterious, especially because the risk to benefit ratio we offer is rather narrow thus far. Inherent to this are the issues of who to treat and the need for predictors of restenosis. After all, it would seem unreasonable to expose all angioplasty patients to possible risks these new approaches may carry.

SUMMARY

Coronary restenosis has proven to be the "Achilles heel" of percutaneous coronary interventions, frequently leading to repeated procedures. The pathogenesis of restenosis can be divided into four phases: early elasic recoil (hours to days), mural thrombus formation (hours to days), neointimal proliferation and extracellular matrix formation (weeks), and chronic geometric arterial changes (months). Restenosis is device nonspecific except for intravascular stents, which can eliminate elastic recoil and prevent geometric vessel changes, leading to decreased restenosis. Of all antithrombotics tried so far, only an inhibitor of the platelet IIb/IIIa integrin, which may lead to early vessel wall passivation, has shown reduction of clinical restenosis. Trapidil (antiproliferative agent) and angiopeptin (somatostatin analog) have also resulted in improved restenosis rates. The field of local drug delivery is currently under investigation in association with radiation or molecular therapy. The current specific target of these approaches is the neointimal proliferation, especially because this is the most dominant mechanism of restenosis after stent placement. Evaluation of these novel methods is complex and interrelates the delivery system with the therapeutic agent administered. However, they provide the means for very specific and timely interruption of the pathogenetic process that may lead to better understanding and, ultimately, elimination of restenosis.

REFERENCES

1. Mueller RL, Sanborn TA. The history of interventional cardiology: cardiac catheterization, angioplasty, and related interventions. Am Heart J 1995;129:146-72.
2. Waller BF, Pinkerton CA, Orr CM, Slack JD, Van Tassel JW, Peters T. Restenosis 1 to 24 months after clinically successful coronary balloon angioplasty: a necropsy study of 20 patients. J Am Coll Cardiol 1991;17:58-70B.
3. Nobuyoshi M, Kimura T, Nosaka H, Mioka S, Ueno K, Yokoi H, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: serial angiographic follow-up of 229 patients. J Am Coll Cardiol 1988;12:616-23.
4. Beatt KJ, Serruys PW, Luijten HE, Rensing BJ, Suryapranata H, de-Feuter P, et al. Restenosis after coronary angioplasty: the paradox of increased lumen diameter and restenosis. J Am Coll Cardiol 1992; 19:258-66.
5. Kuntz RE, Baim DS. Defining coronary restenosis. Newer clinical and angiographic paradigms. Circulation 1993;88:1310-23.
6. Kuntz RE, Gibson CM, Nobuyoshi M, Baim DS. Generalized model of restenosis after conventional balloon angioplasty, stenting and directional atherectomy. J Am Coll Cardiol 1993;21:15-25.
7. Rodriguez A, Santaera O, Larribeau M, Sosa MI, Palacios IF. Early decrease in minimal luminal diameter after successful percutaneous transluminal coronary angioplasty predicts late restenosis. Am J Cardiol 1993;71:1391-5.
8. Fuster V, Falk E, Fallon JT, Badimon L, Chesebro JH, Badimon JJ. The three processes leading to post-PTCA restenosis: dependence the lesion substrate. Thromb Haemost 1995;74:552-9.
9. Chesebro JH, Lam JYT, Badimon L, Fuster V. Restenosis after arterial angioplasty: a hemorrheologic response to injury. Am J Cardiol 1987;60:10-6B.
10. Willerson JT, Yao SK, McNatt J, Benedict CR, Anderson HV, Golino P, et al. Frequency and severity of cyclic flow alternations and platelet aggregation predict the severity of neointimal proliferation following

experimental coronary stenosis and endothelial injury. Proc Natl Acad Sci USA 1991;88:10624-8.
11. Ip JH, Fuster V, Israel D, Badimon L, Badimon J, Chesebro JH. The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J Am Coll Cardiol 1991;17:77-88B.
12. Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. J Clin Invest 1990;86:385-91.
13. Bar-Shavit R, Eldor A, Vlodavsky I. Binding of thrombin to subendothelial extracellular matrix. Protection and expression of functional properties. J Clin Invest 1989;84:1096-104.
14. Moreno P, Falk E, Palacios I, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. Circulation 1994;90:775-8.
15. Casscells W. Migration of smooth muscle and endothelial cells. Critical events in restenosis. Circulation 1992;86:723-9.
16. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. Lab Invest 1987;49:327-33.
17. Ferns GAA, Raines EW, Sprugel K, Motari A, Reidy M, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 1991;253:1129-32.
18. Riessen R, Isner JM, Blessing E, Loushin C, Nikol S, Wight TN. Regional differences in the distribution of the proteoglycans biglycan and decorin in the extracellular matrix of atherosclerotic and restenotic human coronary arteries. Am J Pathol 1994;144:962-74.
19. Nikol S, Isner JM, Pickering JG, Kearney M, Leclerc G, Weir L. Expression of transforming growth factor-B1 is increased in human vascular restenosis lesions. J Clin Invest 1992;90:1582-92.
20. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory enlargement of human atherosclerotic coronary arteries. N Engl J Med 1987;316:1371-5.
21. Losordo DW, Rosenfield K, Kaufman J, Pieczek A, Isner JM. Focal compensatory enlargement of human arteries in response to progressive atherosclerosis. Circulation 1994;89:2570-7.
22. Mintz GS, Popma JJ, Pichard AD. Mechanisms of late arterial responses to transcatheter therapy: a serial quantitative angiographic and intravascular study. Circulation 1994;90(Part 2):I24.
23. Mintz GS, Chuang YC, Popma JJ, Pichard AD, Kent KM, Satler LF, et al. The final percentage cross sectional narrowing (residual plaque burden) is the strongest intravascular ultrasound predictor of angiographic restenosis. J Am Coll Cardiol 1995;25:35A.
24. Pompa JJ, Topol EJ. Factors influencing restenosis after coronary angioplasty. Am J Med 1990;88:1-16N.
25. Hillegass WB, Ohman EM, Califf RM. Restenosis: the clinical issues. In: Topol EJ, editor. Textbook of interventional cardiology, 2nd ed. Philadelphia: W.B. Saunders, 1994:415-35.
26. deGroote P, Bauters C, McFadden EP, Lablanche JM, Leroy F, Bertrand ME. Local lesion-related factors and restenosis after coronary angioplasty: evidence from a quantitative angiographic study in patients with unstable angina undergoing double-vessel angioplasty. Circulation 1995;91:968-72.
27. Desmarais RL, Sarembock IJ, Ayers CR, Vernon SM, Powers ER, Gimple LW. Elevated serum lipoprotein(a) is a risk factor for clinical recurrence after coronary balloon angioplasty. Circulation 1995;91:1403-9.
28. Hirshfeld JW, Schwartz JS, Jugo R, Macdonald RG, Goldberg S, Savage MP, et al. and the M-Heart Investigatorr. Restenosis after coronary angioplasty: a multivariate statistical model to relate lesion and procedure variables to restenosis. J Am Coll Cardiol 1991;18:647-56.
29. Violaris AG, Melkert R, Serruys PW. Long-term luminal renarrowing after successful elective coronary angioplasty of total occlusions. Circulation 1995;91:2140-50.
30. Serruys PW, deJaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndickx G, et al. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. N Engl J Med 1994;331:489-95.
31. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, et al. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. N Engl J Med 1994;331:496-501.
32. Mintz GS, Pichard AD, Kent KM, Satler LF, Popma J, Wong SC, et al. Endovascular stents reduce restenosis by eliminating geometric arterial remodelling: a serial intravascular ultrasound study. J Am Coll Cardiol 1995;25:36A.
33. Colombo A, Hall P, Nakamura S, Almagor Y, Maiello L, Martini G, et al. Intracoronary stenting without anticoagulation accomplished with intravascular ultrasound guidance. Circulation 1995;91:1676-88.
34. Raizner AE, Hollman J, Abukhalil J, Demke D for the Ciprostene Investigators. Ciprostene for restenosis revisited: quantitative analysis of angiograms. J Am Coll Cardiol 1993;21:321A.
35. The EPIC Investigators. Use of monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. N Engl J Med 1994;331:956-61.
36. Harrington RA, Marchant K, Kleiman NS, Leimberger JD, Tcheng JE, Sigmon KN, et al. for the IMPACT Investigators. Bleeding associated with use of a platelet glycoprotein IIb/IIIa inhibitor during routine coronary angioplasty: is too much heparin the culprit? J Am Coll Cardiol 1994;23:106A.
37. Topol EJ, Califf RM, Weisman HF, Ellis SG, Tcheng JE, Worley S, et al. on behalf of the EPIC Investigators. Randomised trial of coronary intervention with antibody against platelet IIb/IIIa integrin for reduction of clinical restenosis: results at six months. Lancet 1994;343:881-6.
38. Ellis SG, Roubin GS, Wilentz J, Douglas JS, King SB. Effect of 18- to 24-hour heparin administration for prevention of restenosis after uncomplicated coronary angioplasty. Am Heart J 1989;117:777-84.
39. Lefkovits J, Topol E. Direct thrombin inhibitors in cardiovascular medicine. Circulation 1994;90:1522-36.
40. Serruys PW, Herrman JPR, Simon R, Rutsch W, Bode C, Laarman GJ, et al. for the HELVETICA Investigators. A comparison of hirudin with heparin in the prevention of restenosis after coronary angioplasty. N Engl J Med 1995;333:757-63.
41. Faxon DP, Spiro TE, Minor S, Cote G, Douglas J, Gottlieb R, et al. and the ERA Investigators. Low molecular weight heparin in prevention of restenosis after angioplasty. Results of enoxaparin restenosis (ERA) trial. Circulation 1994;90:908-14.
42. Nunes GL, Hanson SR, King SB, Sahatjian RA, Scott NA. Local delivery of a synthetic antithrombin with a hydrogel-coated angioplasty balloon catheter inhibits platelet-dependent thrombosis. J Am Coll Cardiol 1994;23:1578-83.
43. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 1992;355:564-6.
44. Weintraub, WS, Boccuzzi SJ, Klein JL, Kosinski AS, King SB, Ivanhoe R, et al. and the Lovastatin Restenosis Trial Study Group. Lack of effect of lovastatin on restenosis after coronary angioplasty. N Engl J Med 1994;331:1331-7.
45. Maresta A, Balducelli M, Cantini L, Casari A, Chioin R, Fabbri M, et al. Trapidil (triazolopyrimidine), a platelet-derived growth factor antagonist, reduces restenosis after percutaneous transluminal coronary angioplasty. Results of the randomized, double-blind STARC Study. Circulation 1994;90:2710-5.
46. Emanuelsson H, Beatt KJ, Bagger JP, Balcon R, Heikkila J, Piessens J, et al. for the European Angiopeptin Study Group. Long-term effects of angiopeptin treatment in coronary angioplasty. Reduction of clinical events but not angiographic restenosis. Circulation 1995;91:1689-96.
47. Serruys PW, Klein W, Tijssen JPG, Rutsch W, Heyndrickx GR, Emanuelsson H, et al. Evaluation of ketanserin in the prevention of restenosis after percutaneous transluminal coronary angioplasty. A multicenter randomized double-blind placebo-controlled trial. Circulation 1993;88:1588-1601.
48. Powell JS, Clozel J, Muller RKM, Kuhn H, Hefti F, Hosang M, et al. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Science 1989;245:186-8.
49. van der Giessen WJ, Hardhammar PA, van Beusekom HMM, et al. Prevention of (sub)acute thrombosis using heparin-coated stents. Circulation 1994;90(part 4):I650.
50. Lincoff AM, Topol EJ, Ellis SG. Local drug delivery for the prevention of restenosis. Fact, fancy, and future. Circulation 1994;90:2070-84.
51. Simons M, Edelman ER, DeKayser JL, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. Nature 1992;359:67-70.

52. Epstein SE, Speir E, Unger EF, Guzman RJ, Finkel T. The basis of molecular strategies for treating coronary restenosis after angioplasty. J Am Coll Cardiol 1994;23:1278-88.
53. Ohno T, Gordon D, San H, Pompili VJ, Imperiale MJ, Nabel GJ, et al. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. Science 1994;265:781-4.
54. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, et al. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. Science 1995;267:518-22.
55. Epstein SE, Speir E, Finkel T. Do antisense approaches to the problem of restenosis make sense? Circulation 1994;88:1351-3.
56. Morishita R, Gibbons GH, Ellison KE, Nakajima M, Zhang L, Kaneda Y, et al. Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. Proc Natl Acad Sci USA 1993;90:8474-8.
57. Pickering JG, Bacha PA, Weir L, Jekanowski J, Nichols JC, Isner JM. Prevention of smooth muscle cell outgrowth from human atherosclerotic plaque by a recombinant cytotoxin specific for the epidermal growth factor receptor. J Clin Invest 1993;91:724-9.
58. Ortu P, laMuraglia GM, Roberts WG, Flotte TJ, Hasan T. Photodynamic therapy of arteries. A novel approach for treatment of experimental intimal hyperplasia. Circulation 1992;85:1189-93.

Heparin Is an Adhesive Ligand for the Leukocyte Integrin Mac-1 (CD11b/CD18)

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Abstract. Previous studies have demonstrated that the leukocyte integrin Mac-1 adheres to several cell surface and soluble ligands including intercellular adhesion molecule-1, fibrinogen, iC3b, and factor X. However, experiments with Mac-1–expressing transfectants, purified Mac-1, and mAbs to Mac-1 indicate the existence of additional ligands. In this paper, we demonstrate a direct interaction between Mac-1 and heparan sulfate glycans. Heparin affinity resins immunoprecipitate Mac-1, and neutrophils and transfectant cells that express Mac-1 bind to heparin and heparan sulfate, but not to other sulfated glycosaminoglycans. Inhibition studies with mAbs and chemically modified forms of heparin suggest the I domain as a recognition site on Mac-1 for heparin, and suggest that either N- or O-sulfation is sufficient for heparin to bind efficiently to Mac-1. Under conditions of continuous flow in which heparins and E-selectin are cosubstrates, neutrophils tether to E-selectin and form firm adhesions through a Mac-1–heparin interaction.

In many immune responses, neutrophils are among the first cells to exit from the circulatory system and traffic to an inflammatory site. For this to occur, neutrophils must attach to endothelial cells, change shape, diapedese, and migrate. Over the past few years, several families of surface receptors on the neutrophil and endothelial cell surface that facilitate movement from the bloodstream have been identified. Selectins form transient attachments that allow neutrophil rolling on inflamed endothelium (11, 66). G-protein–coupled receptors bind soluble inflammatory molecules (25, 66), and signal integrins to strengthen attachment to endothelial cells, and initiate movement to the underlying tissue (11, 66). The details of neutrophil migration to the target site are less clear. A chemotactic gradient of soluble or tethered inflammatory peptides or lipids augments the adhesiveness of surface receptors and reorganizes the cytoskeleton. To move directionally, neutrophils adhere transiently to molecules in the extracellular environment; although the matrix contains an abundant number of adhesive proteins, proteoglycans, and carbohydrates, it is unknown which participate in chemotaxis.

The leukocyte integrins comprise a subfamily of related cell-surface glycoproteins that coordinate adhesive functions including leukocyte migration (12, 65). The members of this family include lymphocyte function–associated antigen-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18). They share a common β subunit that is noncovalently associated with unique, but closely related, α subunits (32). The α subunits share two features in their extracellular region, a 200–amino acid inserted I domain and three tandem EF handlike putative divalent cation-binding repeats (32).

Mac-1 is expressed primarily on myeloid cells. Experiments with blocking mAbs and with neutrophils from patients with a genetic deficiency of leukocyte integrins demonstrate a role for Mac-1 in an array of adhesive interactions that include myeloid cell adhesion to, and transmigration across, endothelium or epithelium, neutrophil homotypic adhesion and chemotaxis, myeloid cell adhesion to serum-coated substrates, and the binding and phagocytosis of opsonized particles (4, 8, 52, 54, 63, 72). Mac-1 sustains these interactions by binding to several cell surface and soluble ligands including intercellular adhesion molecule (ICAM)-1[1], fibrinogen, iC3b, and factor X (1, 2, 8, 19, 63, 72, 73). Recent experiments with mAbs, integrin chimeras, and soluble protein fragments define the 200–amino acid I domain on Mac-1 as a recognition site for at least three of its ligands (21, 70, 75).

Although these four ligands subserve many Mac-1–dependent adhesive functions, they do not account for the entirety of its adhesive interactions. For example, a recep-

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1. *Abbreviations used in this paper:* fMLP, formylmethionylleucylphenylalanine; ICAM, intercellular adhesion molecule; LAD, leukocyte adhesion deficiency.

tor on resting endothelial cells and epithelial cells that lack ICAM-1 has been suggested, but not yet identified (19, 39, 52). A counterreceptor on neutrophils that participates in Mac-1–dependent homotypic adhesion remains postulated, but uncharacterized (4, 54). Finally, the chemotaxis of neutrophils requires Mac-1, but no extracellular matrix ligand has been documented. Several groups, including our own, have attempted unsuccessfully to raise blocking mAbs or to identify novel cDNAs that confer adhesion to purified or cellular Mac-1. Moreover, a recent study suggests that the adhesion of bone marrow cells to stromal fibroblasts may occur, in part through a heparan sulfate interaction with Mac-1 (13). In this report, we document heparin and heparan sulfate as glycosaminoglycan, adhesive ligands for the leukocyte integrin Mac-1. Using data from static and shear flow adhesion assays we suggest novel adhesive pathways through which Mac-1–heparin interactions may mediate neutrophil trafficking from the circulatory system to an inflammatory site.

Materials and Methods

Glycosaminoglycans

High molecular weight (mol wt = 13,000–15,000) porcine intestine mucosal heparin and low molecular weight (mol wt = 5,000) bovine intestine mucosal heparin were purchased from Calbiochem Corp. (La Jolla, CA). Chemically modified forms of heparin were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Heparan sulfate (mol wt = 7,500) from bovine intestinal mucosa, keratan sulfate from bovine cornea, and all forms of shark cartilage chondroitin sulfate (A, B, C) were purchased from Sigma Chemical Co. (St. Louis, MO).

mAbs

The following mAbs were used from ascites: TS1/22 (anti-CD11a) (60), Mn41 (anti-CD11b) (22), LPM19c (anti-CD11b) (69), 44a (anti-CD11b) (5), OKM9 (anti-CD11b) (72), TMG-65 (anti-CD11b) (69), 14B6E.2 (anti-CD11b) (69), the CBRM1 series (anti-CD11b: M1/1, M1/2, M1/4, M1/9, M1/10, M1/13, M1/16, M1/17, M1/18, M1/20, M1/21, M1/22, M1/23, M1/24, M1/25, M1/26, M1/27, M1/28, M1/29, M1/30, M1/31, M1/32, M1/33, M1/34) (21), CBRp150,95/4G1 (anti-CD11c) (67), and TS1/18 (anti-CD18) (60). The following mAbs were used as purified IgG: W6/32 (anti-HLA A, B, C) (7), OKM1 (anti-CD11b) (72), LM2/1 (anti-CD11b) (45), RJ5.7 (anti-CD18, a gift of Dr. R. Rothlein, Boehringer Ingelheim Ltd., Ridgefield, CT) (23), and DREG-56 (anti-L-selectin, a gift of Dr. T. Kishimoto, Boehringer Ingelheim Ltd.) (33).

Tissue Culture, Transfection, and Cell Preparation

Peripheral blood neutrophils from healthy volunteers or patients with leukocyte adhesion deficiency (LAD) were isolated from whole venous blood by dextran sedimentation, Ficoll gradient centrifugation, and hypotonic lysis (46). Before experimentation, cells were maintained at room temperature in HBSS, 10 mM Hepes, pH 7.3, and 1 mM $MgCl_2$ in polypropylene tubes (2097; Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ). In some experiments, neutrophils were maintained in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

CHO cell stable transfectants that express Mac-1, p150,95 or ICAM-1 have been described (21). These cells were maintained in α-MEM, 10% dialyzed FCS, 16 μM thymidine, 0.05 μM methotrexate, 2 mM glutamine, and 50 μg/ml gentamicin.

Protein Purification and SDS Gel Electrophoresis

As described (51), the procedure for isolating cytochrome b559 and Mac-1 includes solubilizing stimulated granulocyte membranes in 2% *n*-octyl-β-D-glucopyranoside followed by passage of the detergent extract over a column of wheat germ agglutinin–Sepharose 4B. After washing, the bound proteins were eluted from the wheat germ agglutinin–Sepharose 4B with a membrane resuspension buffer (100 mM KCl, 10 mM NaCl, 10 mM Hepes, pH 7.3, 1 mM EDTA, 1 mM PMSF, 10 μg/ml chymostatin, 200 mM *N*-acetyl glucosamine, 200 μg/ml chitobiose, 0.4 M NaCl, and 0.2% Triton X-100). The eluate was concentrated and diluted 10-fold to reduce the salt concentration to 50 mM, and passed over a 5-ml column of heparin-Ultrogel (LKB Instruments Inc., Bromma, Sweden). Cytochrome b559 and Mac-1 were eluted from the heparin column with a gradient of increasing NaCl containing 0.1% Triton X-100. Peak heparin eluate fractions were subjected to SDS-PAGE on linear polyacrylamide gradient gels (8–16%) followed by either staining or electrophoretic transfer onto nitrocellulose as described (51).

The purification of Mac-1 by LM2/1 immunoaffinity chromatography has been described (19). Approximately 2–5 μg of purified Mac-1 in 300 mM NaCl, 50 mM triethanolamine, 0.1 M Tris, 1% *n*-octyl-β-D-glucopyranoside, pH 8.0, was diluted 20-fold with 10 mM Hepes, pH 7.4, 0.1% Triton X-100, 35 mM NaCl, and incubated for 3 h at 4°C with either 50 μl of heparin–Ultrogel or, as a control, Ultrogel alone. After washing with 40 mM NaCl, 10 mM KCl, 10 mM Hepes, pH 7.4, 0.1% Triton X-100, the beads were eluted for 1 h at 4°C with 0.5 ml of 400 mM NaCl, 100 mM KCl, 10 mM Hepes, pH 7.4, and 0.1% Triton X-100. The eluates were concentrated 10-fold using a 30-kD cutoff microconcentrating device (Amicon Corp., Danvers, MA) and subjected to SDS-PAGE followed by silver staining as described above.

Static Adhesion Assays

The neutrophil binding assay was based on a previously published protocol (20). Purified heparins (5 mg/ml in PBS), chondroitin sulfates (A, B, or C, 5 mg/ml in PBS), or fibrinogen (2 mg/ml in PBS; Sigma Chemical Co.) were spotted (25 μl) onto 6-cm bacterial petri dishes (1007; Fisher Scientific Co., Pittsburgh, PA) for 90 min at room temperature. Protein or glycosaminoglycan was removed, and the plates were blocked with the detergent Tween 20. Neutrophils (3×10^6 cells in 1 ml) were resuspended in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, and for some experiments, preincubated with mAbs (1/200 dilution of ascites or 20 μg/ml purified mAb) or with soluble glycosaminoglycan for 15 min at room temperature. In some experiments, 1 mM $CaCl_2$ was added to the HBSS binding buffer. Subsequently, cells were added to the petri dishes in the presence of formylmethionylleucylphenylalanine (fMLP) (10^{-7} M, final volume of 3 ml) and allowed to adhere for 3.5 min. Nonadherent cells were removed, and binding was quantitated as described (20, 21).

The binding of CHO cell transfectants is a modification of a previously described protocol (21). Heparins and chondroitin sulfates were adsorbed to 6-cm petri dishes. After a 90-min incubation at room temperature, nonspecific binding sites were blocked with a 0.5% heat-treated BSA solution (PBS, 1 mM $MgCl_2$, 0.025% NaN_3). CHO cell transfectants, after dejachment from tissue culture plates with HBSS, 10 mM Hepes, pH 7.3, 5 mM EDTA, were washed twice, resuspended (8×10^5 cells/ml) in HBSS, 10 mM Hepes, pH 7.3, 1 mM $MgCl_2$, 0.5% heat-treated BSA, and bound to glycosaminoglycan-coated petri dishes for 20 min at room temperature. Nonadherent cells were removed, and binding was quantitated as described (21). In some experiments, the number of nonviable, adherent transfectants was determined by staining with PBS, 0.4% trypan blue, and this value was subtracted.

Laminar Flow Adhesion Assays

Purified heparin (5 mg/ml) or heparan sulfate (10 mg/ml) was spotted on bacterial petri dishes (Nunc Inc., Naperville, IL) and blocked with Tween 20 as described above. Control substrates were prepared by blocking dishes solely with Tween 20. Soluble recombinant E-selectin (36) (a generous gift of Dr. R. Lobb, Biogen, Cambridge, MA) was diluted (0.4 μg/ml in 50 mM $NaHCO_3$, pH 9.1) and absorbed on bacterial petri dishes for 2 h at room temperature. The substrate was washed twice with PBS, and heparin (5 mg/ml) or control PBS solution was adsorbed onto the E-selectin–coated substrate for 10 h at room temperature. The substrate was then blocked with 0.2% Tween 20, PBS for 60 min at room temperature.

The laminar flow assays were performed in a parallel wall flow chamber as described previously (35, 36). Briefly, a petri dish slide on which purified heparin, heparan sulfate, and/or recombinant E-selectin had been absorbed was assembled as the lower wall of the parallel flow chamber and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon Inc., Instrument Division, Garden City, NY). Cells that tethered or arrested were quantitated by analysis of images videotaped with a video camera (TEC-470 CCD; Optronics Engineering, Goleta, CA) and Hi 8 recorder (CVD-1000; Sony Corp., Park Ridge, NJ). Peripheral blood neutrophils were resuspended (5×10^5 cells/ml) in binding media

(HBSS, 10 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2 mM $CaCl_2$), in the absence or presence of IL-8 (50 ng/ml, Genzyme Corp., Cambridge, MA) and immediately perfused through the chamber at different flow rates to obtain the indicated shear stresses at the chamber wall. In experiments with a single divalent cation, neutrophils were washed in HBSS, 10 mM Hepes, pH 7.4, 10 mM EDTA and resuspended in HBSS, 10 mM Hepes, pH 7.4, 2 mM $CaCl_2$.

For mAb inhibition studies, neutrophils (10^7 cells/ml) were incubated in HBSS, 10 mM Hepes, pH 7.4 for 5 min at room temperature with 30 μg/ml of purified mAbs (CBRM1/29, DREG-56, W6/32) or a 1:30 dilution of ascites (CBRM1/34). The cell suspension was diluted in a 20-fold volume of binding media and immediately perfused into the flow chamber. In some experiments, to assess the requirement of active metabolism on integrin-mediated adhesion, cells were preincubated for 5 min in binding media supplemented with 0.5% NaN_3.

Neutrophil binding to heparin or control substrates at subphysiologic shear stress was determined by counting the number of cells that attached over a 3-min period of continuous shear flow for a given field of view (0.43 mm^2). Detachment assays were performed on cells tethered at subphysiologic shear flow to purified heparin. The shear flow was increased every 10 s to a maximum of 14.6 dyn/cm^2, in 2–2.5-fold increments and the number of cells remaining bound at the end of each 10-s interval was determined.

Neutrophil tethering to E-selectin substrate coimmobilized with heparin was performed under physiologic shear flow (1.05 dyn/cm^2), and the rate of cell tethering (number of events lasting at least 2 s/min per 0.43 mm^2 field) was determined. No tethering was observed under these conditions to substrates coated with heparin alone. Rolling velocities of neutrophils (30–35 per field) that tethered under flow were determined at a shear stress of 1.5 dyn/cm^2 by cell displacements measured over 5-s intervals. For comparison of effects of mAbs or chemical treatments, identical fields were used for binding experiments to ensure that results reflected uniform site density of immobilized adhesive molecules.

Results

Previously, we identified ICAM-1 on endothelial cells as a counterreceptor for Mac-1 (19). In the course of these experiments, we observed that unstimulated endothelial cells bind to purified Mac-1 even in the presence of blocking mAbs to ICAM-1. Since Mac-1 does not bind to ICAM-2 (15, 19), we postulated the existence of an additional adhesive ligand on endothelium for Mac-1. Subsequently, we found several mesenchymally derived tumor cell lines (e.g., RD 3/5 and FS 12/3) that lack or had low expression of ICAM-1, ICAM-2, and ICAM-3 (15, 16), but adhere strongly to purified Mac-1 (Diamond, M. S., and T. A. Springer, unpublished observations). Because we were unable to clone a cDNA or make function-blocking antibodies against this putative ligand, we hypothesized a highly conserved molecule. Two candidates were proteoglycans and sialylated carbohydrates, molecules that function broadly in cell adhesion in the extracellular matrix and on the cell surface.

While purifying cytochrome b559 from solubilized granulocytes, a "contaminating" 150-kD protein was observed that eluted from a heparin affinity resin under conditions of moderately high ionic strength (250 mM NaCl; Fig. 1, lane *4*) (51). Because of the similarity of molecular weight to Mac-1, this eluate was immunoblotted. Both mAbs (Fig. 1, lane *3*) and polyclonal antisera (data not shown) against CD11b identified the 150-kD protein as a subunit of Mac-1. Additionally, functionally active Mac-1 binds to immobilized heparin directly; Mac-1 purified by immunoaffinity chromatography (19) is reprecipitated by heparin-Ultrogel (Fig. 1, lane *5*), but not by Ultrogel alone (Fig. 1, lane *7*). Scanning densitometry showed that 73% of the purified Mac-1 bound to Heparin-Ultrogel (data not shown).

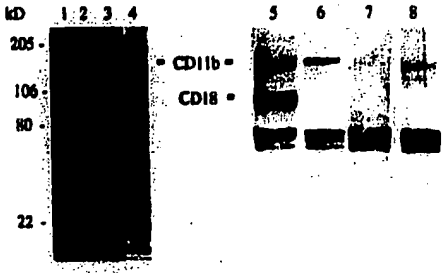

Figure 1. Immunoblotting and SDS 8–16% gradient PAGE of Mac-1 purified from detergent-solubilized granulocyte lysates by immunoaffinity or heparin affinity chromatography. Lane *1*, Mac-1 purified by mAb (LM2/1) affinity chromatography, immunoblotted with anti-Mac-1 mAb (44a), and visualized with peroxidase-conjugated goat anti-mouse IgG; lane *2*, Mac-1 purified by mAb (LM2/1) affinity chromatography, immunoblotted with negative control mAb, and visualized with peroxidase-conjugated goat anti-mouse; lane *3*, immunoblot with anti-Mac-1 mAb (44a) of the peak heparin eluate fraction from the purification of granulocyte cytochrome b559 (see Materials and Methods), and visualized with peroxidase-conjugated goat anti-mouse IgG; lane *4*, Coomassie stain of SDS-PAGE of peak heparin eluate fraction shown as an immunoblot in lane *3*; lanes *5–8*: silver staining of reduced SDS-PAGE of purified Mac-1 after binding and elution from heparin-Ultrogel (see Materials and Methods): lane *5*, Mac-1 eluted (400 mM NaCl) from heparin-Ultrogel showing the characteristic 150 and 95 kD subunits; lane *6*, SDS denaturation of heparin-Ultrogel matrix after high salt elution of Mac-1 demonstrating minimal residual integrin on the matrix; lane *7*, Control high salt (400 mM NaCl) eluate from Ultrogel after incubation with purified Mac-1 demonstrating a lack of nonspecific binding of the two subunits of Mac-1; lane *8*, SDS denaturation of the Ultrogel matrix after high salt elution demonstrating no residual integrin on the matrix.

To assess the significance of this biochemical interaction between Mac-1 and heparin, we assayed neutrophil adhesion to different purified glycosaminoglycans. Resting neutrophils bind weakly to both low (mol wt = 5,000) and high (mol wt = 13,000–15,000) molecular weight forms of heparin, but not to chondroitin sulfate A, B, or C (Fig. 2, and data not shown). Stimulation with the chemotactic peptide fMLP enhances adhesion of neutrophils to heparin, but not to chondroitin sulfate; in parallel experiments, an equivalent number of activated neutrophils bind to ICAM-1 and fibrinogen (data not shown). To confirm that Mac-1 on neutrophils mediates the adhesion to heparin, we tested neutrophils from patients who have the genetic disease LAD which is characterized by an absence of Mac-1 on leukocytes (3). LAD patient neutrophils do not adhere to any form of heparin or chondroitin sulfate in the absence or presence of fMLP (Fig. 2) or in buffers that contain only Mg^{2+} or both Mg^{2+} and Ca^{2+} (data not shown).

Inhibition experiments with mAbs sustain the role of Mac-1 in neutrophil adhesion to heparin. Blocking mAbs to Mac-1 (LPM19c) but not to LFA-1 (TS1/22), p150,95 (CBRp150,95/4G1), or L-selectin (DREG-56) abolish adhesion to heparin (Fig. 3; see Fig. 7). A panel of mAbs to Mac-1 for which epitopes have been mapped on the α subunit, and which have been tested for inhibition of binding to other ligands (21), was used to localize the recognition site for heparin (Table I). mAbs to the I domain inhibited

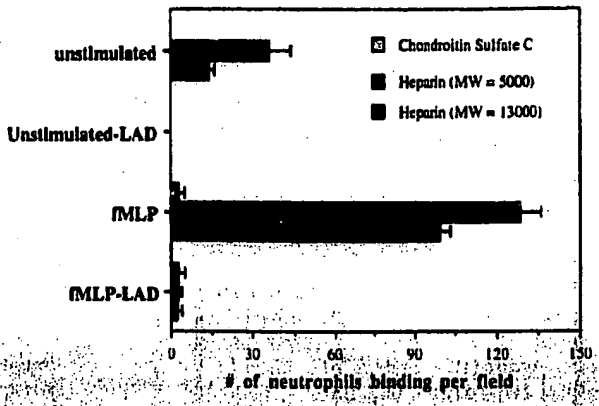

Figure 2. Neutrophil adhesion to different glycosaminoglycans. Peripheral blood neutrophils from healthy volunteers or LAD patients were resuspended (4×10^6 cells/ml) and added to 60-mm petri dishes coated with spots of chondroitin sulfate C, or low or high molecular weight heparin. Binding in the absence or presence of 10^{-7} M fMLP was for 3.5 min at room temperature, and unbound cells were removed by 10 washes with a Pasteur pipette. Bound cells were quantitated by visually scoring the number of cells in five microscopic fields (×100). One representative experiment of four is shown, and bars indicate standard deviations.

binding with a mean of 57.3 ± 24.4%, whereas mAbs to the COOH-terminal region blocked with a mean of 11.0 ± 9.8%. 5 of 18 mAbs that mapped to the I domain blocked strongly (>80%), whereas no mAb that localized to the COOH-terminal region showed >30% inhibition. The one mAb (CBRM1/20) that mapped directly to the divalent cation-binding region had little inhibitory effect. The one mAb that mapped to sites in both the NH_2-terminal and divalent cation-binding regions blocked 63% of the binding. Collectively, these data suggest a recognition site in the I domain on Mac-1 for heparin.

To eliminate the possibility that mAbs to Mac-1 inhibit neutrophil adhesion by an indirect effect, we examined

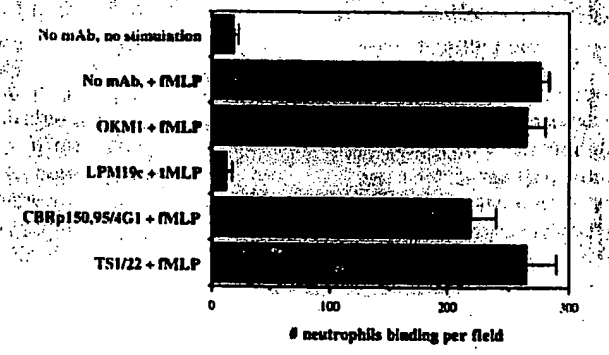

Figure 3. The effect of mAb on neutrophil adhesion to high molecular weight heparin. Peripheral blood neutrophils were resuspended (4×10^6 cells/ml), preincubated at room temperature for 15 min with mAbs, and added to 60-mm petri dishes coated with heparin in the absence or presence of 10^{-7} M fMLP. The cell-binding assay, washing, and quantitation were performed as described in Fig. 2. mAbs: Media (*no mAb*), anti-Mac-1 (*OKM1, LPM19c*), anti-p150,95 (*CBRp150,95/4G1*), anti-LFA-1 (*TS1/22*). One representative experiment of three is shown, and bars indicate standard deviations.

Table I. Summary of Inhibition of Neutrophil Adhesion to Heparin by mAbs and Comparison with Structural Epitope

mAb	Epitope	Percent inhibition ± SEM
LPM19c	I domain	94.1 ± 1
OKM9	I domain	45.4 ± 2
LM2/1	I domain	29.1 ± 9
TMG-65	I domain	82.0 ± 3
Mn41	I domain	84.5 ± 9
14B6E.2	I domain	28.8 ± 8
CBRM1/1	I domain	57.4 ± 8
CBRM1/2	I domain	41.6 ± 7
CBRM1/4	I domain	27.8 ± 11
CBRM1/13	I domain	8.5 ± 13
CBRM1/21	I domain	73.3 ± 15
CBRM1/22	I domain	64.2 ± 13
CBRM1/24	I domain	34.4 ± 14
CBRM1/27	I domain	86.3 ± 9
CBRM1/29	I domain	69.6 ± 8
CBRM1/31	I domain	60.5 ± 8
CBRM1/33	I domain	55.7 ± 9
CBRM1/34	I domain	88.0 ± 5
OKM1	COOH-terminal	2.8 ± 14
CBRM1/9	COOH-terminal	−6.2 ± 13
CBRM1/10	COOH-terminal	24.0 ± 5
CBRM1/16	COOH-terminal	10.8 ± 10
CBRM1/17	COOH-terminal	10.8 ± 15
CBRM1/18	COOH-terminal	9.8 ± 17
CBRM1/23	COOH-terminal	6.1 ± 1
CBRM1/25	COOH-terminal	3.7 ± 5
CBRM1/26	COOH-terminal	22.2 ± 17
CBRM1/30	COOH-terminal	26.4 ± 6
CBRM1/32	NH_2-cation	62.8 ± 13
CBRM1/20	cation	13.1 ± 2
CBRM1/28	?	12.3 ± 6
CBRp150,95/4G1	p150,95	−3.3 ± 10
TS1/18	CD18	16.8 ± 0.5
R15.7	CD18	60.2 ± 7

Neutrophils were prepared as described in Fig. 3. Results are the average of three to five independent experiments. Epitopes are assigned according to their binding to a series of Mac-1/p150,95 chimeras as described (21).

transfectant cell binding to heparin. CHO cells that express Mac-1 adhere strongly to heparin but not to chondroitin sulfate (Fig. 4 *A*); adhesion is specific as mAbs to Mac-1, but not to LFA-1 block (Fig. 4 *B*). In contrast, CHO cells that express ICAM-1 do not bind to heparin. Thus, Mac-1 is both necessary and sufficient for attachment to heparin. Interestingly, CHO cells that express p150,95 bind to heparin, although at lower efficiency.

To evaluate the interaction more quantitatively, we tested soluble heparin for its ability to inhibit Mac-1–dependent neutrophil adhesion to immobilized heparin (Fig. 5). Preincubation of neutrophils with soluble heparin dose dependently inhibited adhesion to heparin (inhibition constant $[K_i] = 9\ \mu M$) but not to fibrinogen or iC3b-coated erythrocytes (data not shown). In contrast, soluble chondroitin sulfate C, which also bears a strong negative charge secondary to sulfation, had no effect on Mac-1–dependent neutrophil adhesion to heparin or fibrinogen over a wide range of concentrations.

Because heparins are a heterogeneous group of molecules with differences in carbohydrate backbone and extent of sulfation (38), we tested how neutrophils adhere to a series of chemically modified heparins (Fig. 6). Most hep-

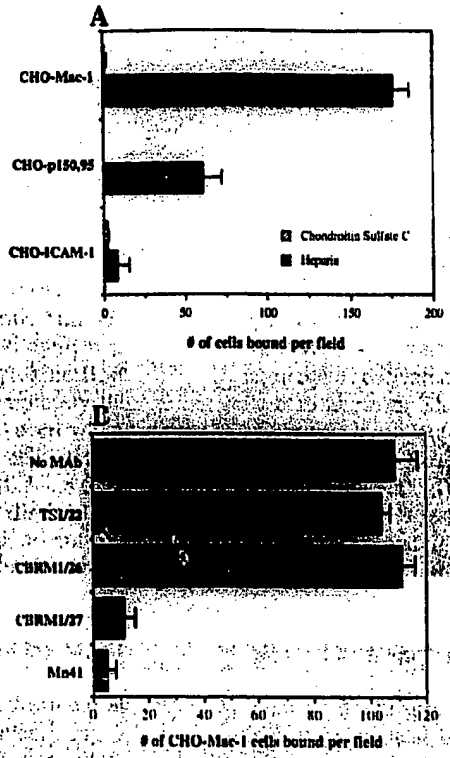

Figure 4. Binding of CHO cell transfectants to high molecular weight heparin. (*A*) Mac-1, p150,95 and ICAM-1–expressing CHO cells were detached, resuspended (8×10^5 cells) in 1 ml, and bound to 60-mm petri dishes coated with spots of heparin and chondroitin sulfate C for 20 min at room temperature. Unbound cells were removed after five washes with a Pasteur pipette, and binding was quantitated as described in Fig. 2. One representative experiment of three is shown, and bars indicate standard deviations. (*B*) Mac-expressing CHO cells were prepared and assayed as in *A* for binding to heparin in the presence of no mAb, mAb to LFA-1 (*TS1/22*), or mAb to Mac-1 (*CBRM1/26, CBRM1/27, Mn41*). One representative experiment of three is shown, and bars indicate standard deviations.

arins are N-sulfated at the free amino group of GlcNAc, and O-sulfated at C-6 of GlcNAc and at C-2 of IdoA. However, some heparins show additional O-sulfation at the C-2 and C-3 of GlcA or the C3 of GlcNAc. Activated neutrophils adhere poorly to heparins that are completely desulfated (CDSNAc). Sulfation at the N-position of the glucosamine residue of a completely desulfated heparin (CDSNS) restores adhesion. Although this suggests an important role for the N-sulfate group, additional motifs must be critical because N-desulfated forms of heparin (NDSNAc) sustain neutrophil adhesion.

Thus far, static adhesion assays have been used to define an interaction between Mac-1 and heparin. Because closely related moieties (e.g., heparan sulfate containing proteoglycans) are expressed on endothelial cell surfaces in vivo, we examined how neutrophils interact with immobilized heparin under conditions of continuous laminar flow. At low, subphysiologic shear stress (0.36 dyn/cm^2), neutrophils activated with the inflammatory cytokine IL-8 bind to heparin. The adhesion is blocked completely by mAbs to Mac-1, but not L-selectin (Fig. 7 *A*), even when 20–40% of the L-selectin is retained on the cell surface (data not shown). At physiologic shear stress (0.7–0.8 dyn/cm^2), neutrophil attachment to heparin is diminished. Resting or activated neutrophils, once adhered, do not roll on heparin at any shear stress examined. In contrast, neutrophil rolling is supported by immobilized E- or P-selectins (35, 36), and by the L-selectin ligand, peripheral node in (37). Much like its interaction with ICAM-1 (35), Mac-1 binds to heparin at subphysiologic shear flows, and attachment results in spreading and resistance to detachment at high physiological shear stresses (e.g., 15 dyn/cm^2, Fig. 7 *B*). Similarly, heparan sulfate supports Mac-1–dependent firm adhesions that are highly resistant to detaching shear forces (Fig. 7 *C*). In the presence of Ca^{2+} alone, no neutrophil tethering or rolling on heparan sulfate under physiologic or subphysiologic shear flow was observed.

Because Mac-1 on neutrophils does not attach to heparin under physiologically relevant flow conditions, we questioned whether an initial tethering interaction via E-selectin could deaccelerate a neutrophil enough to facilitate a Mac-1–heparin interaction. To dissect the different modes of adhesion, tethering rates and the shear resistance of adhesion were measured for a substrate adsorbed with E-selectin alone or coadsorbed with E-selectin and heparin (Fig. 8). Neutrophils bind to E-selectin via counterreceptors that are decorated with sialyl Le^x (34). E-selectin sustains neutrophil tethering, but does not support arrest at physiologic shear stresses (36). Experiments were performed in buffers containing Ca^{2+} only, Ca^{2+} and Mg^{2+}, and in the presence or absence of blocking mAb to Mac-1.

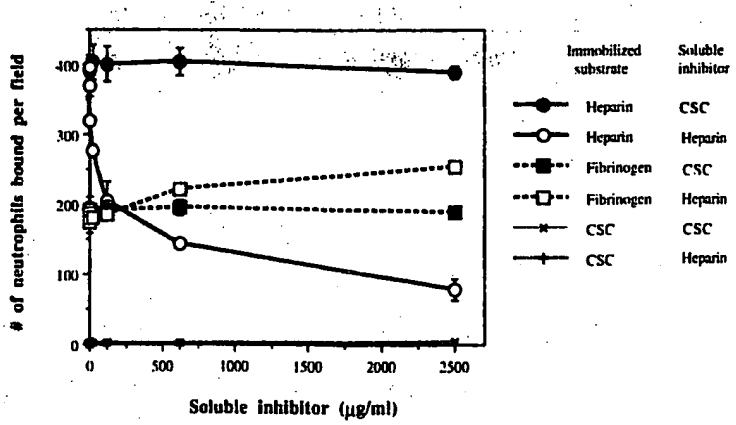

Figure 5. Neutrophil adhesion to fibrinogen, high molecular weight heparin, and chondroitin sulfate C in the presence of soluble high molecular weight heparin or chondroitin sulfate C. Peripheral blood neutrophils were resuspended (4×10^6 cells/ml), preincubated at room temperature for 15 min with varying concentrations of soluble heparin or chondroitin sulfate C, and added to 60-mm petri dishes coated with fibrinogen, heparin, and chondroitin sulfate C in the presence of 10^{-7} M fMLP. The cell-binding assay, washing, and quantitation were performed as described in Fig. 2. One representative experiment of three is shown, and bars indicate standard deviations.

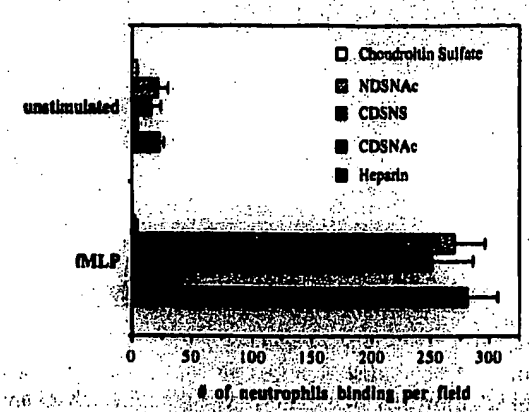

Figure 6. Neutrophil adhesion to chemically modified derivatives of heparin and chondroitin sulfate C. Peripheral blood neutrophils were resuspended (4×10^6 cells/ml), preincubated at room temperature for 15 min with mAbs, and added to 60-mm petri dishes coated with chemically modified forms of heparin (*NDSNAc*, N-desulfated, N-acetylated; *CDSNS*, completely desulfated, N-sulfated; CDSNAc, completely desulfated, N-acetylated) and chondroitin sulfate C in the absence or presence of 10^{-7} M fMLP. The cell-binding assay, washing, and quantitation were performed as described in Fig. 2. One representative experiment of three is shown, and bars indicate standard deviations.

selectins require Ca^{2+}, and leukocyte integrins require Mg^{2+} for adhesion to their respective ligands (28, 34). Under conditions of physiological shear stress (1.0 dyn/cm^2), neutrophils do not tether on heparin substrates or on E-selectin–heparin substrates in the presence of mAbs to E-selectin (data not shown). Ca^{2+} is required for neutrophil tethering on E-selectin (Fig. 8 *A*), but no firm adhesion or neutrophil arrest develops on selectins even in the presence of Mg^{2+} (data not shown and 36). The addition of Mg^{2+} does not affect the rate of tethering to E-selectin. When heparin is coadsorbed with E-selectin, Ca^{2+} enables optimal tethering and rolling as the addition of Mg^{2+} does not augment the tethering rate (Fig. 8 *A*). Mg^{2+}, however, increases the efficiency of events that occur after tethering and rolling. In the absence of mAbs to Mac-1, the arrest of tethered and rolling neutrophils, and hence accumulation of cells, is increased by the addition of Mg^{2+} on an E-selectin–heparin substrate, but not on E-selectin alone (data not shown); arrest occurred immediately upon tethering or after a brief period of rolling. Nonarrested neutrophils roll at identical velocities on E-selectin or E-selectin–heparin substrates in the presence of Ca^{2+} or Ca^{2+}/Mg^{2+}, with or without blocking mAbs to Mac-1. Finally, if neutrophils attach to an E-selectin–heparin substrate in the presence of both divalent cations at physiologic shear stresses, they form firm adhesions and do not detach at elevated shear stresses unless the Mac-1 component is blocked (Fig. 8 *B*). Collectively, these experiments distinguish a Mac-1–heparin interaction from neutrophil rolling on selectins, and suggest that neutrophil rolling on E-selectin at physiological shear is required so that a second, firm attachment between Mac-1 and a heparin ligand will occur, resulting in cell arrest.

Discussion

In this paper, we describe a receptor–ligand interaction between the leukocyte integrin Mac-1 and heparins. The following evidence was obtained for this interaction: (*a*) Mac-1 that is isolated from human granulocytes directly binds to heparin affinity resins; (*b*) neutrophils that are activated with fMLP or IL-8 bind to heparins, but not to other sulfated glycosaminoglycans; (*c*) LAD patient neutrophils that lack surface expression of Mac-1 do not bind heparin; (*d*) mAbs to Mac-1, but neither lymphocyte function–associated antigen-1 nor L-selectin inhibit neutrophil attachment to heparin or heparan sulfate; and (*e*) CHO cells that express Mac-1 bind strongly to heparin, and this adhesion is inhibited specifically by mAbs.

Although interactions with carbohydrates have not been characterized widely for integrins, previous reports indicated that Mac-1 might recognize polysaccharide determinants. Mac-1 appears to bind carbohydrate moieties on yeast β glucan and bacterial lipopolysaccharide (56, 57, 74), and more recently, heparin or heparan sulfate has been suggested to interact with Mac-1 (13). This latter study showed that soluble heparins and enzymatic treatment with heparinase inhibited the binding of CD45 and Mac-1 in A4 bone marrow–derived cell lysates to Swiss 3T3 fibroblasts (13); however, no direct adhesion to heparin by cells that express Mac-1 was documented. In contrast, we demonstrate specific adhesion to heparin of neutrophils and transfectants that express Mac-1, and no adhesion to heparin with LAD neutrophils that lack Mac-1 but express CD45 (62, and data not shown).

To localize the recognition site of heparin on Mac-1, mAbs to defined structural epitopes were assayed for their capacity to inhibit adhesion. This technique previously predicted the 200–amino acid I domain as a recognition site for ICAM-1, fibrinogen, and iC3b (21). Subsequent investigations with recombinant I domain polypeptides confirm that this region participates in ligand recognition (44, 70, 75). Inhibition experiments with mAbs suggest that heparin interacts with Mac-1 through at least one site in the I domain. Because fewer I domain mAbs (27% compared to 67% for iC3b, ICAM-1, and fibrinogen) strongly block adhesion to heparin, the binding site may be smaller than that for other characterized Mac-1 ligands. It appears that the recognition site for heparin may be distinct or partially overlapping with respect to other Mac-1 ligands. Two mAbs (14B6E.2, CBRM1/4), that abolish binding to iC3b, ICAM-1, and fibrinogen, do not affect binding to heparin. One mAb (CBRM1/27) that poorly inhibits adhesion to ICAM-1 and iC3b blocks adhesion to heparin, and soluble heparin does not reduce Mac-1–dependent neutrophil adhesion to fibrinogen.

Transfectant studies in CHO cells suggest a heparin interaction with p150,95 that is lower affinity than with Mac-1. The p150,95 molecule is expressed at substantially lower levels than Mac-1 on neutrophils, and this lower expression, or greater resistance to activation by fMLP and IL-8, may explain the dominant role of Mac-1 on neutrophils in interacting with heparin. Mac-1 and p150,95 already share several ligands including fibrinogen (40), iC3b (10, 47, 55), and ICAM-1 (21). Some mesenchymal cell lines that lack ICAM expression bind to purified Mac-1 and p150,95 but not to lymphocyte function–associated antigen-1 (Diamond, M. A., and T. A. Springer, unpublished observations).

Previous studies have suggested that L-selectin binds

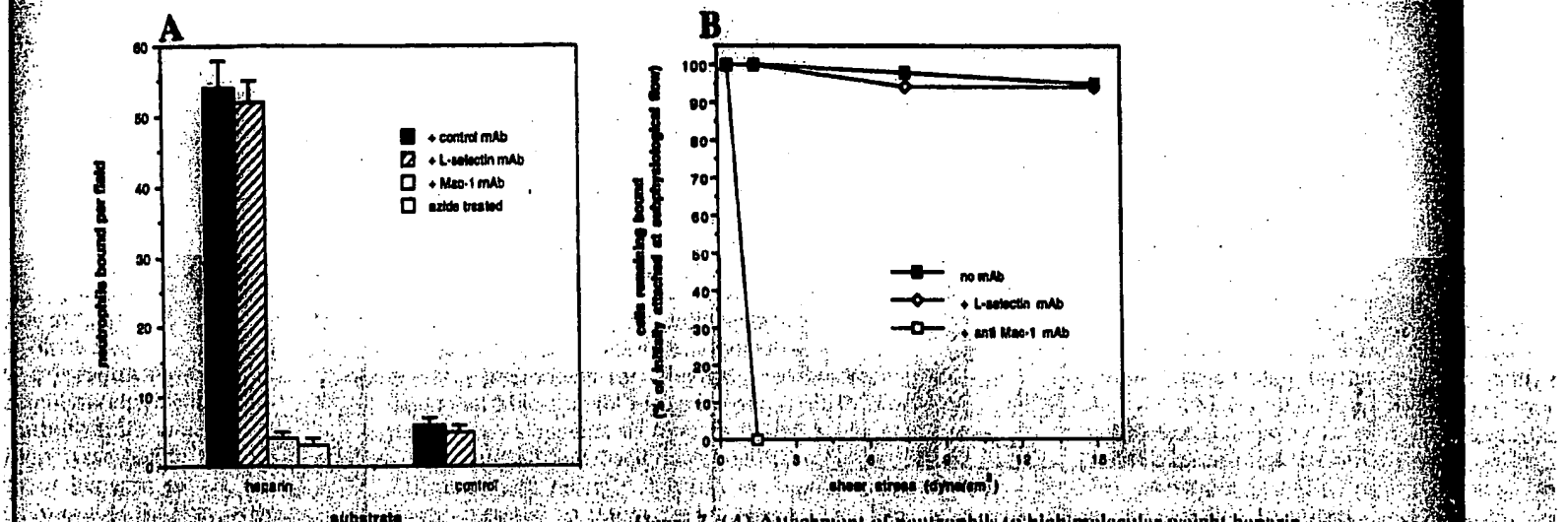

Figure 7. (*A*) Attachment of neutrophils to high molecular weight heparin under laminar flow. Peripheral blood neutrophils (2×10^7 cells/ml) were preincubated at room temperature for 5 min in HBSS binding media with mAbs (anti–Mac-1 (CBRM1/29); anti–L-selectin (DREG-56)), diluted 20-fold in binding media containing Ca^{2+} and Mg^{2+}, and allowed to adhere to a heparin- or control-coated substrate in the presence of IL-8 (50 ng/ml) during flow at a subphysiologic shear stress of 0.36 dyn/cm². After 3 min of continuous flow adherent neutrophils were quantitated. Data are averaged from two experiments, and bars indicate range. In some experiments, the neutrophils were pretreated with NaN_3 as described in Materials and Methods. (*B*) Detachment assay after attachment of neutrophils to heparin. Neutrophils were resuspended (5×10^6 cells/ml), preincubated at room temperature for 5 min with mAbs (anti–Mac-1 (CBRM1/29); anti–L-selectin (DREG-56)), and allowed to attach to heparin in the presence of IL-8 (50 ng/ml) at low flow (0.36 dyn/cm²) for 3 min. Subsequently, shear stress was applied in staged increments. Neutrophils bound after 10 s at each indicated shear are expressed as the percentage of neutrophils that settled initially on heparin. All adherent neutrophils remained stationary at all shear stresses. One representative experiment of three is shown. (*C*) Detachment assay after adhesion of neutrophils to heparan sulfate. Neutrophils (10^6/ml) were perfused at low shear flow (0.15 dyn/cm²) and allowed to adhere to heparan sulfate coated substrates for 2 min in medium containing the indicated divalent cations. In some experiments, neutrophils were preincubated at room temperature for 5 min with a mAb to Mac-1 (CBRM1/34). Shear stress was increased in staged increments and cells remaining bound were quantitated as described in *B*. Number of cells bound to a field at each indicated shear stress is shown. One experiment of three is shown.

heparin-like molecules (48, 50), yet we do not observe a significant contribution of this interaction in either of our assays. In the presence of Ca^{2+} but not Mg^{2+}, resting neutrophils which express high levels of functional L-selectin do not interact with heparin or heparan sulfate under static or flow conditions (Fig. 7 *C*, and data not shown). mAbs to L-selectin do not inhibit neutrophil adhesion to heparin or heparan sulfate (data not shown) and LAD patient neutrophils that lack Mac-1 but express L-selectin do not bind heparin. Furthermore, neutrophil attachment to heparin improves after stimulation with fMLP, a condition that prompts rapid shedding of L-selectin (31). Even when L-selectin is retained on the neutrophil surface, mAbs to Mac-1 fully abrogate the formation of firm adhesions on heparin and heparan sulfate. Finally, the adhesion to heparin is energy and temperature dependent (data not shown), requires Mg^{2+} and low shear stress flow conditions for attachment, and is not rolling in nature.

The interaction between Mac-1 and heparin is equivalent in strength (K_i of adhesion = 9 μM) to many other integrin–ligand pairs (18), and the level of binding is similar to that of two other described Mac-1 ligands, ICAM-1 and fibrinogen (19, 21). Complete chemical desulfation of heparin significantly reduces binding to Mac-1, whereas N-sulfation of the amino group of GlcNAc restores adhesion. Thus, the presence of at least one type of sulfate group on heparin appears crucial for its interaction with Mac-1. Because forms of heparin that lack N-sulfation (NDSNAc) retain the ability to bind Mac-1, secondary O-sulfated moieties, and carbohydrate structures probably contribute to the recognition site. Furthermore, heparan sulfate sustains Mac-1–dependent adhesion of neutrophils; heparan sulfate shows a lower proportion of N-sulfated GlcNAc residues and a lower overall degree of O-sulfation (38). Since Mac-1 does not interact with chondroitin sulfates, keratan sulfate, or hyaluronic acid (data not shown), the interaction with heparan sulfate glycosaminoglycans is specific, and does not reflect nonspecific binding to sulfate groups or other highly negatively charged structures.

Although we demonstrate an in vitro adhesive interac-

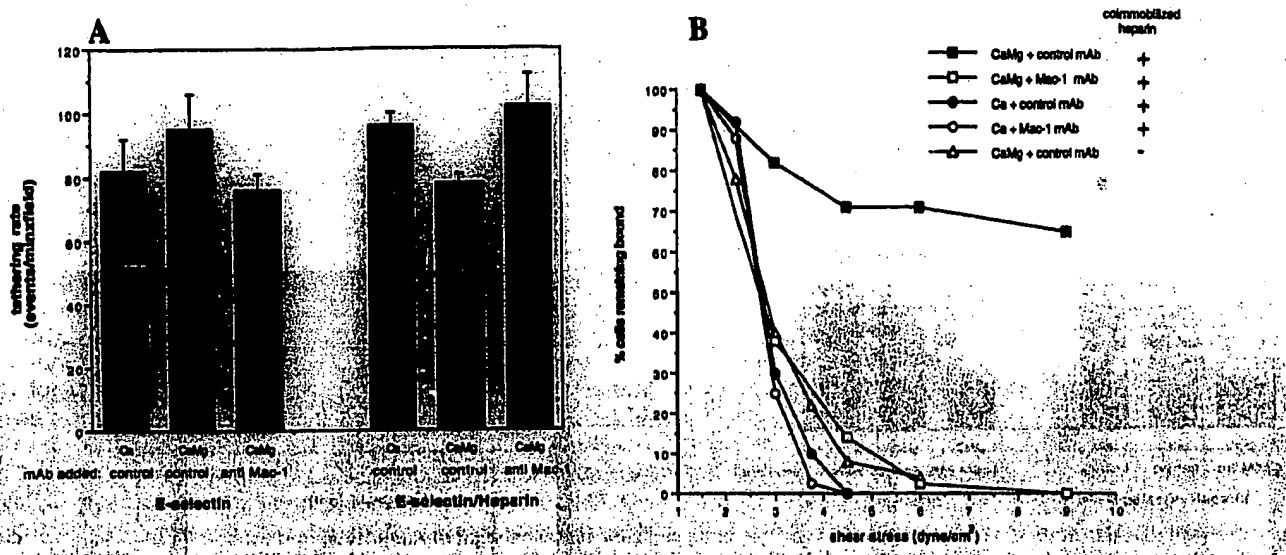

Figure 8. The effect of divalent cations on rolling and firm adhesions of neutrophils tethered to E-selectin–heparin substrate under physiological shear flow. Neutrophils were suspended in a HBSS buffer containing 2 mM Ca^{2+} alone, or with 1 mM Mg^{2+} and perfused through a flow chamber at a shear of 1.05 dyn/cm². (*A*) Neutrophil tethering events (cell attachments of at least 2 s duration) were determined on substrates adsorbed with recombinant E-selectin alone or with heparin. Tethering is expressed as the number of tethering events per minute per field (0.43 mm² in area). Cells were preincubated with anti–Mac-1 mAb (CBRM1/34) or control anti-HLA class I mAb (W6/32) for 5 min before perfusion into the chamber. Results are expressed as the mean ± range. (*B*) After 1 min of cell perfusion at a shear of 1.05 dyn/cm² that allowed accumulation of tethered cells, the flow rate was increased, and the number of cells that remained bound to the substrate was measured. The data is expressed as the percentage of the initially bound neutrophils. For blocking experiments, cells were preincubated with mAbs to Mac-1 and HLA class I as described above. The control mAb had no effect on neutrophil tethering, rolling, or arrest. Results in *A* and *B* are representative of three separate experiments.

tion between purified heparins and Mac-1, the form to which Mac-1 binds in vivo remains unknown. Physiologically, heparins decorate proteoglycans, molecules that function broadly in cell adhesion and communication (9, 58). Many cell surface and matrix-associated proteoglycans contain heparan sulfate, a less sulfated form of heparin (38, 58), whereas those found in intracellular granules of some leukocytes contain the more highly sulfated heparin molecule (68). Candidate proteoglycan ligands that display heparan sulfate include syndecans, perlecan, and glypican (14, 42, 49, 59). Syndecans are an evolutionarily related gene family of four transmembrane proteoglycans, and are expressed broadly in epithelial, endothelial, connective, and neural tissues (30). A common ancestral gene family member in *Drosophila* colocalizes with integrins at sites of muscle attachment during wing morphogenesis (26, 64). Antimicrobial peptides generated during wound repair induce expression of syndecans on endothelial cells, fibroblasts, and in the surrounding extracellular matrix of granulation tissue (24). Perlecan is a basal lamina transmembrane proteoglycan involved in neovascularization (6), and has been suggested to interact with both β_1 and β_3 integrins in a heparin-dependent manner (27). Glypican is a heparan sulfate proteoglycan that is membrane linked through a glycosyl phosphatidylinositol anchor (14), and is expressed on aortic and umbilical vein endothelial cells (43). Future studies will assess which of the described heparan sulfate proteoglycans serve as adhesive ligands for Mac-1. Preliminary studies indicate a high level of specificity of Mac-1 and heparan sulfate proteoglycan interactions; the purified extracellular domain of syndecan-1 (29), which is expressed primarily on epithelial cells (30), does not support neutrophil adhesion (Alon, R., and T. A. Springer, unpublished observations).

Where could a Mac-1–heparin interaction be important? It may contribute to neutrophil migration through the endothelium during inflammation. The laminar flow experiments suggest that Mac-1 does not make transient interactions with heparin or heparan sulfate that characterize rolling; at physiological shear stresses, activated or resting neutrophils do not attach to heparin substrates. In contrast, at conditions of low shear stress, activated neutrophils attach to and spread on heparins in a Mac-1–dependent manner, similar to that observed on ICAM-1 (35). At high shear flows, when E-selectin is coadsorbed with heparin, neutrophils make transient rolling attachments via selectins, and then arrest and develop high shear resistance through a heparin–Mac-1 interaction. We have no evidence for an overlap between selectin- and Mac-1–mediated adhesion to heparin; Mac-1 binding to heparin does not support neutrophil rolling. Instead, it enables the firm adhesion of cells that have been slowed physiologically by selectin-mediated rolling, or allowed sufficient contact time experimentally by binding at subphysiologic flow. Because they bind inflammatory cytokines and chemokines such as IL-8 (66, 71), heparins can both present molecules that activate Mac-1 and serve as ligands for adhesion and spreading. Thus, heparin moieties may complement other receptors such as ICAM-1 in the Mac-1–mediated neutrophil extravasation process.

Another role for a Mac-1–heparin interaction may be in neutrophil migration after extravasation. Heparan sulfates

resent in the extracellular matrix as components of ted proteoglycans, and may serve as adhesive tracks migration and haptotaxis of leukocytes that express c-1. In addition, heparin-containing proteoglycans re- e within primary granules of resting neutrophils (53). fter activation, these molecules translocate to the surface nd into the surrounding environment. A chemotactic timulus in the periphery may induce neutrophils to acti- vate Mac-1 and directionally secrete an adhesive ligand to create a path for locomotion.

A Mac-1–heparin interaction also may explain the phenomenon of neutrophil homotypic aggregation. Activation by high concentrations of certain chemotactic factors (e.g., fMLP) induces neutrophil degranulation, activation of surface Mac-1 (17), and a Mac-1–dependent homotypic adhesion (54). Because neutrophil granules contain proteoglycans, such as serglycin, that display polyvalent heparin-like moieties (41, 53), degranulation may promote homotypic aggregation by bridging Mac-1 on adjacent cells. Interestingly, neutrophil aggregation does not occur at 16°C, a temperature that prevents degranulation, but is permissive for the activation of neutrophil Mac-1 (61, and Diamond, M. S., and T. A. Springer, unpublished observations).

In this report we have described a specific interaction between heparin, heparan sulfate, and the leukocyte integrin Mac-1. Future studies must aim at identifying the precise functional side chain on heparan sulfate glycans that binds to Mac-1, the proteoglycans that are decorated with this moiety, and the biological context for Mac-1–heparin adhesion. Such information may enable the generation of heparin or heparan sulfate analogues that serve selectively as antiinflammatory agents in vivo.

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References

1. Altieri, D. C., and T. S. Edgington. 1988. The saturatable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-1 receptor. *J. Biol. Chem.* 263:7007–7015.
2. Altieri, D. C., R. Bader, P. M. Mannucci, and T. S. Edgington. 1988. Oligospecificity of the cellular adhesion receptor MAC-1 encompasses an inducible recognition specificity for fibrinogen. *J. Cell Biol.* 107:1893–1900.
3. Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu. Rev. Med.* 38:175–194.
4. Anderson, D. C., L. J. Miller, F. C. Schmalsteig, R. Rothlein, and T. A. Springer. 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure–function assessment employment subunit-specific monoclonal antibodies. *J. Immunol.* 137: 15–27.
5. Arnaout, M. A., R. F. Todd, III, N. Dana, J. Melamed, S. F. Schlossman, and H. R. Colten. 1983. Inhibition of phagocytosis of complement C3– or immunoglobulin G–coated particles and of C3bi binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein (Mo1). *J. Clin. Invest.* 72:171–179.
6. Aviezer, D., D. Hecht, M. Safran, M. Elsinger, G. David, and A. Yayon. 1994. Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor–receptor binding, mitogenesis, and angiogenesis. *Cell.* 79: 1005–1013.
7. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA, and other human cell surface antigens—new tools for genetic analysis. *Cell.* 14:9–20.
8. Beller, D. I., T. A. Springer, and R. D. Schreiber. 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med.* 156:1000–1009.
9. Bernfield, M., R. Kokenyesi, M. Kato, M. T. Hinkes, J. Spring, R. L. Gallo, and E. J. Lose. 1992. Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell. Biol.* 8:365–393.
10. Bilsland, C. A., M. S. Diamond, and T. A. Springer. 1994. The leukocyte integrin p150,95 (CD11c/CD18) acts as a receptor for iC3b. Activation by a heterologous β subunit and localization of a ligand recognition site to the I domain. *J. Immunol.* 152:4582–4589.
11. Butcher, E. C. 1991. Leukocyte–endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell.* 67:1033–1036.
12. Carlos, T. M., and J. M. Harlan. 1990. Membrane proteins involved in phagocyte adherence to endothelium. *Immunol. Rev.* 114:1–24.
13. Coombe, D. R., S. M. Watt, and C. R. Parish. 1994. Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. *Blood.* 84:739–752.
14. David, G., V. Lories, B. Decock, P. Marynen, J. J. Cassiman, and H. Van den Berghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111:3165–3176.
15. de Fougerolles, A. R., S. A. Stacker, R. Schwarting, and T. A. Springer. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *J. Exp. Med.* 174:253–267.
16. de Fougerolles, A. R., L. B. Klickstein, and T. A. Springer. 1993. Cloning and expression of intercellular adhesion molecule 3 reveals a strong homology to other immunoglobulin family counter-receptors for lymphocyte function-associated antigen 1. *J. Exp. Med.* 177:1187–1192.
17. Diamond, M. S., and T. A. Springer. 1993. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J. Cell Biol.* 120:545–556.
18. Diamond, M. S., and T. A. Springer. 1994. The dynamic regulation of integrin adhesiveness. *Curr. Biol.* 4:506–517.
19. Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer. 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J. Cell Biol.* 111:3129–3139.
20. Diamond, M. S., D. E. Staunton, S. D. Marlin, and T. A. Springer. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third Ig-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell.* 65:961–971.
21. Diamond, M. S., J. Garcia-Aguilar, J. K. Bickford, A. L. Corbi, and T. A. Springer. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J. Cell Biol.* 120:1031–1043.
22. Eddy, A., S. L. Newman, F. Cosio, T. LeBien, and A. Michael. 1984. The distribution of the CR3 receptor on human cells and tissue as revealed by a monoclonal antibody. *Clin. Immunol. Immunopathol.* 31:371–389.
23. Entman, M. L., K. Youker, S. B. Shappell, C. Siegel, R. Rothlein, W. J. Dreyer, F. C. Schmalstieg, and C. W. Smith. 1990. Neutrophil adherence to isolated adult canine myocytes: evidence for a CD18-dependent mechanism. *J. Clin. Invest.* 85:1497–1506.
24. Gallo, R. L., M. Ono, T. Povsic, C. Page, E. Eriksson, M. Klagsbrun, and M. Bernfield. 1994. Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl. Acad. Sci. USA.* 91:11035–11039.
25. Gerard, C., and N. P. Gerard. 1994. The pro-inflammatory seven transmembrane segment receptors of the leukocyte. *Curr. Opin. Immunol.* 6: 140–145.
26. Gotwals, P. J., S. E. Paine-Saunders, K. A. Stark, and R. O. Hynes. 1994. Drosophila integrins and their ligands. *Curr. Opin. Cell Biol.* 6:734–739.
27. Hayashi, K., J. A. Madri, and P. D. Yurchenco. 1992. Endothelial cells interact with the core protein of basement membrane perlecan through β1 and β3 integrins: an adhesion modulated by glycosaminoglycan. *J. Cell Biol.* 119:945–959.
28. Hynes, R. O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* 69:11–25.
29. Kato, M., H. Wang, M. Bernfield, J. T. Gallagher, and J. E. Turnbull. 1994. Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *J. Biol. Chem.* 269:18881–18890.
30. Kim, C. W., O. A. Goldberger, R. L. Gallo, and M. Bernfield. 1994. Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns. *Mol. Biol. Cell.* 5:797–805.
31. Kishimoto, T. K., M. A. Jutila, E. L. Berg, and E. C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins are inversely regulated by chemotactic factors. *Science (Wash. DC).* 245:1238–1241.
32. Kishimoto, T. K., R. S. Larson, A. L. Corbi, M. L. Dustin, D. E. Staunton, and T. A. Springer. 1989. The leukocyte integrins: LFA-1, Mac-1, and p150,95. *Adv. Immunol.* 46:149–182.
33. Kishimoto, T. K., M. A. Jutila, and E. C. Butcher. 1990. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 87:2244–2248.

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References

1. Altieri, D. C., and T. S. Edgington. 1988. The saturatable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-1 receptor. *J. Biol. Chem.* 263:7007–7015.
2. Altieri, D. C., R. Bader, P. M. Mannucci, and T. S. Edgington. 1988. Oligospecificity of the cellular adhesion receptor MAC-1 encompasses an inducible recognition specificity for fibrinogen. *J. Cell Biol.* 107:1893–1900.
3. Anderson, D. C., and T. A. Springer. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu. Rev. Med.* 38:175–194.
4. Anderson, D. C., L. J. Miller, F. C. Schmalstieg, R. Rothlein, and T. A. Springer. 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessment employment subunit-specific monoclonal antibodies. *J. Immunol.* 137: 15–27.
5. Arnaout, M. A., R. F. Todd, III, N. Dana, J. Melamed, S. F. Schlossman, and H. R. Colten. 1983. Inhibition of phagocytosis of complement C3- or immunoglobulin G–coated particles and of C3bi binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein (Mo1). *J. Clin. Invest.* 72:171–179.
6. Aviezer, D., D. Hecht, M. Safran, M. Elsinger, G. David, and A. Yayon. 1994. Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor–receptor binding, mitogenesis, and angiogenesis. *Cell.* 79: 1005–1013.
7. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA, and other human cell surface antigens—new tools for genetic analysis. *Cell.* 14:9–20.
8. Beller, D. I., T. A. Springer, and R. D. Schreiber. 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med.* 156:1000–1009.
9. Bernfield, M., R. Kokenyesi, M. Kato, M. T. Hinkes, J. Spring, R. L. Gallo, and E. J. Lose. 1992. Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell. Biol.* 8:365–393.
10. Bilsland, C. A., M. S. Diamond, and T. A. Springer. 1994. The leukocyte integrin p150,95 (CD11c/CD18) acts as a receptor for iC3b. Activation by a heterologous β subunit and localization of a ligand recognition site to the I domain. *J. Immunol.* 152:4582–4589.
11. Butcher, E. C. 1991. Leukocyte–endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell.* 67:1033–1036.
12. Carlos, T. M., and J. M. Harlan. 1990. Membrane proteins involved in phagocyte adherence to endothelium. *Immunol. Rev.* 114:1–24.
13. Coombe, D. R., S. M. Watt, and C. R. Parish. 1994. Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. *Blood.* 84:739–752.
14. David, G., V. Lories, B. Decock, P. Marynen, J.-J. Cassiman, and H. Van den Berghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111:3165–3176.
15. de Fougerolles, A. R., S. A. Stacker, R. Schwarting, and T. A. Springer. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *J. Exp. Med.* 174:253–267.
16. de Fougerolles, A. R., L. B. Klickstein, and T. A. Springer. 1993. Cloning and expression of intercellular adhesion molecule 3 reveals a strong homology to other immunoglobulin family counter-receptors for lymphocyte function-associated antigen 1. *J. Exp. Med.* 177:1187–1192.
17. Diamond, M. S., and T. A. Springer. 1993. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J. Cell Biol.* 120:545–556.
18. Diamond, M. S., and T. A. Springer. 1994. The dynamic regulation of integrin adhesiveness. *Curr. Biol.* 4:506–517.
19. Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer. 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J. Cell Biol.* 111:3129–3139.
20. Diamond, M. S., D. E. Staunton, S. D. Marlin, and T. A. Springer. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third Ig-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell.* 65:961–971.
21. Diamond, M. S., J. Garcia-Aguilar, J. K. Bickford, A. L. Corbi, and T. A. Springer. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J. Cell Biol.* 120:1031–1043.
22. Eddy, A., S. L. Newman, F. Cosio, T. LeBien, and A. Michael. 1984. The distribution of the CR3 receptor on human cells and tissue as revealed by a monoclonal antibody. *Clin. Immunol. Immunopathol.* 31:371–389.
23. Entman, M. L., K. Youker, S. B. Shappell, C. Siegel, R. Rothlein, W. J. Dreyer, F. C. Schmalstieg, and C. W. Smith. 1990. Neutrophil adherence to isolated adult canine myocytes: evidence for a CD18-dependent mechanism. *J. Clin. Invest.* 85:1497–1506.
24. Gallo, R. L., M. Ono, T. Povsic, C. Page, E. Eriksson, M. Klagsbrun, and M. Bernfield. 1994. Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl. Acad. Sci. USA.* 91:11035–11039.
25. Gerard, C., and N. P. Gerard. 1994. The pro-inflammatory seven transmembrane segment receptors of the leukocyte. *Curr. Opin. Immunol.* 6: 140–145.
26. Gotwals, P. J., S. E. Paine-Saunders, K. A. Stark, and R. O. Hynes. 1994. Drosophila integrins and their ligands. *Curr. Opin. Cell Biol.* 6:734–739.
27. Hayashi, K., J. A. Madri, and P. D. Yurchenco. 1992. Endothelial cells interact with the core protein of basement membrane perlecan through β1 and β3 integrins: an adhesion modulated by glycosaminoglycan. *J. Cell Biol.* 119:945–959.
28. Hynes, R. O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* 69:11–25.
29. Kato, M., H. Wang, M. Bernfield, J. T. Gallagher, and J. E. Turnbull. 1994. Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *J. Biol. Chem.* 269:18881–18890.
30. Kim, C. W., O. A. Goldberger, R. L. Gallo, and M. Bernfield. 1994. Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns. *Mol. Biol. Cell.* 5:797–805.
31. Kishimoto, T. K., M. A. Jutila, E. L. Berg, and E. C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins are inversely regulated by chemotactic factors. *Science (Wash. DC).* 245:1238–1241.
32. Kishimoto, T. K., R. S. Larson, A. L. Corbi, M. L. Dustin, D. E. Staunton, and T. A. Springer. 1989. The leukocyte integrins: LFA-1, Mac-1, and p150,95. *Adv. Immunol.* 46:149–182.
33. Kishimoto, T. K., M. A. Jutila, and E. C. Butcher. 1990. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 87:2244–2248.

34. Lasky, L. A. 1992. Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science (Wash. DC)*. 258:964–969.
35. Lawrence, M. B., and T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from the prerequisite for adhesion through integrins. *Cell*. 65:859–873.
36. Lawrence, M. B., and T. A. Springer. 1993. Neutrophils roll on E-selectin. *J. Immunol.* 151:6338–6346.
37. Lawrence, M. B., E. L. Berg, E. C. Butcher, and T. A. Springer. 1995. Rolling of lymphocytes and neutrophils on peripheral node addressin and subsequent arrest on ICAM-1 in shear flow. *Eur. J. Immunol.* 25:1025–1031.
38. Lindahl, U., and L. Kjellen. 1991. Heparin or heparan sulfate—what is the difference. *Thromb. Haemostasis*. 66:44–48.
39. Lo, S. K., O. A. Van Seventer, S. M. Levin, and S. D. Wright. 1989. Two leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands. *J. Immunol.* 143:3325–3329.
40. Loike, J. D., B. Sodeik, L. Cao, S. Leucona, J. I. Weitz, P. A. Detmers, S. D. Wright, and S. C. Silverstein. 1991. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the Aα chain on fibrinogen. *Proc. Natl. Acad. Sci. USA*. 88:1044–1048.
41. Mailler, P., P. M. Allisi, M. J. Mihajvila, J. P. Perin, P. Jolika, and F. Bonnet. 1992. Expression of the serglycin gene in human leukemic cell lines. *Leukemia (Baltimore)*. 6:1143–1147.
42. Mali, M., P. Jaakkola, A. Arvilommi, and M. Jalkanen. 1990. Sequence of human syndecan indicates a novel gene family of integral membrane proteoglycans. *J. Biol. Chem.* 265:6884–6889.
43. Mertens, G., J. J. Cassiman, H. Van den Berghe, J. Vermylen, and G. David. 1992. Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. *J. Biol. Chem.* 267:20435–20443.
44. Michishita, M., V. Videm, and M. A. Arnaout. 1993. A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. *Cell*. 72:857–867.
45. Miller, L. J., R. Schwarting, and T. A. Springer. 1986. Regulated expression of the Mac-1, LFA-1, p150,95 glycoprotein family during leukocyte differentiation. *J. Immunol.* 137:2891–2900.
46. Miller, L. J., D. F. Bainton, N. Borregaard, and T. A. Springer. 1987. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. *J. Clin. Invest.* 80:535–544.
47. Myones, B. L., J. G. Dalzell, N. Hogg, and G. D. Ross. 1988. Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR_4) activity resembling CR_3. *J. Clin. Invest.* 82:640–651.
48. Nelson, R. M., O. Cecconi, W. G. Roberts, A. Aruffo, R. J. Linhardt, and M. P. Bevilacqua. 1993. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood*. 82:3253–3258.
49. Noonan, D. M., A. Fuller, P. Valente, S. Cai, E. Horigan, M. Sasaki, Y. Yamada, and J. R. Hassell. 1991. The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein receptor, and the neural cell adhesion molecule. *J. Biol. Chem.* 266:22939–22947.
50. Norgard-Sumnicht, K. E., N. M. Varki, and A. Varki. 1993. Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. *Science (Wash. DC)*. 261:480–483.
51. Parkos, C. A., R. A. Allen, C. G. Cochrane, and A. J. Jesaitis. 1987. Purified cytochrome *b* from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J. Clin. Invest.* 80:732–742.
52. Parkos, C. A., C. Delp, M. A. Arnaout, and J. L. Madara. 1991. Neutrophil migration across a cultured intestinal epithelium: dependence on a CD11b/CD18-mediated event and enhanced efficiency in physiological direction. *J. Clin. Invest.* 88:1605–1612.
53. Parmley, R. T., R. E. Hurst, M. Takagi, S. S. Spicer, and R. L. Austin. 1983. Glycosaminoglycans in human neutrophils and leukemic myeloblasts: ultrastructural, cytochemical, immunologic, and biochemical characterization. *Blood*. 61:257–266.
54. Patarroyo, M., P. G. Beatty, N. Serhan, and C. G. Gahmberg. 1985. Identification of a cell-surface glycoprotein mediating adhesion in human granulocytes. *Scand. J. Immunol.* 22:619–631.
55. Postigo, A. A., A. L. Corbí, F. Sánchez-Madrid, and M. O. De Landázuri. 1991. Regulated expression and function of CD11c/CD18 integrin on human B lymphocytes. Relation between attachment to fibrinogen and triggering of proliferation through CD11c/CD18. *J. Exp. Med.* 174:1313–1322.
56. Ross, G. D., J. A. Cain, and P. J. Lachmann. 1985. Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. *J. Immunol.* 134:3307–3315.
57. Ross, G. D., J. A. Cain, B. L. Myones, S. L. Newman, and P. J. Lachmann. 1987. Specificity of membrane complement receptor type three (CR3) for β-glucans. *Complement*. 4:61–74.
58. Ruoslahti, E. 1988. Structure and biology of proteoglycans. *Annu. Rev. Cell. Biol.* 4:229–255.
59. Saku, T., and H. Furthmayr. 1989. Characterization of the major heparan sulfate proteoglycan secreted by bovine aortic endothelial cells in culture. *J. Biol. Chem.* 264:3514–3523.
60. Sánchez-Madrid, F., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff, and T. A. Springer. 1982. Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. *Proc. Natl. Acad. Sci. USA*. 79:7489–7493.
61. Schleiffenbaum, B., R. Moser, M. Patarroyo, and U. Fehr. 1989. The cell surface glycoprotein Mac-1 (CD11b/CD18) mediates neutrophil adhesion and modulates degranulation independently of its quantitative cell surface expression. *J. Immunol.* 142:3537–3545.
62. Schwinzer, R. 1989. Cluster report: CD45/CD45R. *In* Leucocyte Typing IV: White Cell Differentiation Antigens. W. Knapp, B. Dorken, W. Gilks, E. Rieber, R. Schmidt, H. Stein, and A. van dem Borne, editors. Oxford University Press, Oxford, UK. 628–634.
63. Smith, C. W., S. D. Marlin, R. Rothlein, C. Toman, and D. C. Anderson. 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J. Clin. Invest.* 83:2008–2017.
64. Spring, J., S. E. Paine-Saunders, R. O. Hynes, and M. Bernfield. 1994. Drosophila syndecan: conservation of a cell-surface heparan sulfate proteoglycan. *Proc. Natl. Acad. Sci. USA*. 91:3334–3338.
65. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Wash. DC)*. 346:425–433.
66. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 76:301–314.
67. Stacker, S. A., and T. A. Springer. 1991. Leukocyte integrin p150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J. Immunol.* 146:648–655.
68. Stevens, R. L., K. Otsu, J. H. Web, R. V. Tantravahi, and K. F. Austen. 1987. Co-sedimentation of chondroitin sulfate A glycosaminoglycans and proteoglycans with the cytolytic secretory granules of rat large granular lymphocyte (LGL) tumor cells, and identification of a mRNA in normal and transformed LGL that encodes proteoglycan. *J. Immunol.* 139:863–868.
69. Uciechowski, P., and R. Schmidt. 1989. Cluster report: CD11. *In* Leucocyte Typing IV: White Cell Differentiation Antigens. W. Knapp, B. Dorken, W. Gilks, E. Rieber, R. Schmidt, H. Stein, and A. van dem Borne, editors. Oxford University Press, Oxford, UK. 543–551.
70. Ueda, T., P. Rieu, J. Brayer, and M. A. Arnaout. 1994. Identification of the complement iC3b binding site in the β2 integrin CR3 (CD11b/CD18). *Proc. Natl. Acad. Sci. USA*. 91:10680–10684.
71. Webb, L. M. C., M. U. Ehrengruber, I. Clark-Lewis, M. Baggiolini, and A. Rot. 1993. Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc. Natl. Acad. Sci. USA*. 90:7158–7162.
72. Wright, S. D., P. E. Rao, W. C. Van Vourhis, L. S. Craigmyle, K. Iida, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein. 1983. Identification of the C3bi receptor of human monocytes and macrophages with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*. 80:5699–5703.
73. Wright, S. D., J. I. Weitz, A. J. Huang, S. M. Levin, S. C. Silverstein, and J. D. Loike. 1988. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc. Natl. Acad. Sci. USA*. 85:7734–7738.
74. Wright, S. D., S. M. Levin, M. T. C. Jong, Z. Chad, and L. G. Kabbash. 1989. CR3 (CD11b/CD18) express one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. *J. Exp. Med.* 169:175–183.
75. Zhou, L., D. H. S. Lee, J. Plescia, C. Y. Lau, and D. C. Altieri. 1994. Differential ligand binding specificities of recombinant CD11b/CD18 integrin I domain. *J. Biol. Chem.* 269:17075–17079.

Rapid review

Drug-eluting stents in vascular intervention

Rossella Fattori, Tommaso Piva

Context Restenosis is the most important long-term limitation of stent implantation for coronary artery disease, occurring in 15–60% of patients. In-stent restenosis, a refractory coronary lesion resulting from neointimal hyperplasia, challenges both vascular biologist and interventional cardiologist. Various drugs and devices have been used tried to overcome restenosis but are not particularly successful. Over 1 500 000 percutaneous coronary interventions are done annually. Restenosis is not only important clinically but also for its impact on health-care costs.

Starting point Growth and migration of vascular smooth-muscle cells result in neointimal proliferation after vascular injury and are the key mechanism of in-stent restenosis. The rationale of the most recent approaches to restenosis (eg, brachytherapy and immunosuppressive agents) arises from the similarity between tumour-cell growth and the benign tissue proliferation which characterises intimal hyperplasia. Several immunosuppressants have been tested for their potential to inhibit restenosis, with the novel strategy of administering the drug via a coated stent platform. Local drug delivery achieves higher tissue concentrations of drug without systemic effects, at a precise site and time. The first multicentre trial with stents coated with sirolimus was by Marie-Claude Morice and colleagues (*N Engl J Med* 2002; **346:** 1773–80). In a trial of 238 patients, restenosis of 50% or more at 6 months was 0% and 27% with sirolimus or normal stents ($p<0·001$), respectively, after percutaneous revascularisation. Muzaffer Degertekin and colleagues (*Circulation* 2002; **106:** 1610–13) present data on 2-year follow-up of 15 patients who had been implanted with the sirolimus stent in another study, and confirm persistent inhibition of restenosis and an absence of unexpected adverse events.

Where next? Local application of antiproliferative agents is a promising technique and research is developing. Other agents with potential benefits (eg, statins, local gene-therapy, adenovirus-mediated arterial gene-transfer, L-arginine, abciximab, angiopeptin, recombinant pegylated hirudin, and hiloprost) as well as improvements in polymer technology (biodegradable smart polymers, coatings for multiple-drug release) are under evaluation. The clinical impact of the elimination of restenosis may influence the approach to coronary artery disease, the future of cardiac surgery, and health-care economics in cardiology.

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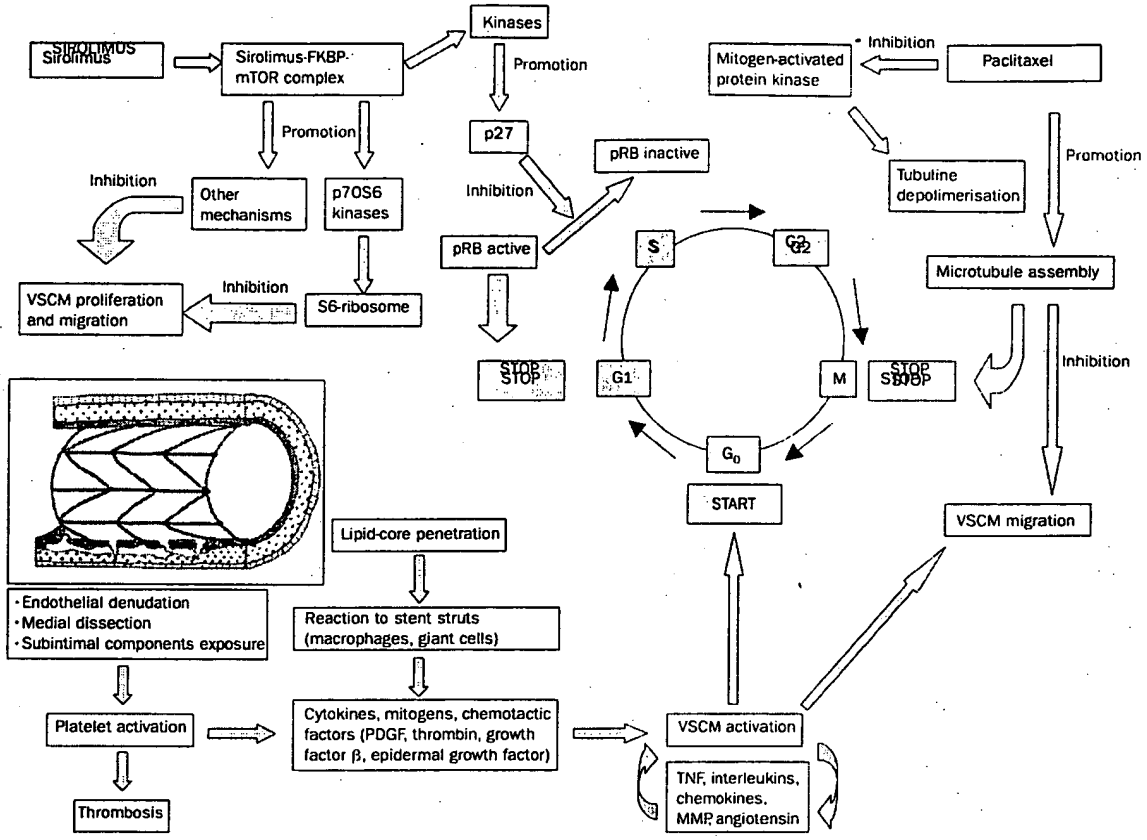
Percutaneous transluminal coronary angioplasty (PTCA) has become the main method of coronary revascularisation, accounting for more than 1 500 000 procedures worldwide every year.[1] Despite technical advancements, of which coronary stenting has been the most significant, restenosis remains the major problem that hampers the procedure's efficacy. Compared with balloon angioplasty alone, coronary stenting coupled with aggressive antithrombotic and antiplatelet therapy improved acute outcome and long-term negative arterial remodelling.[2,3] However, in practice stenting can lead to in-stent restenosis, a particular refractory form of neointimal proliferation. Stent restenosis rates are reported to be 15–20% in ideal coronary lesions, but may occur in over 30–60% of patients with complex lesions (eg, small vessel, diffuse atheromasia, and bifurcation lesion). Moreover recurrent restenosis is more common after percutaneous treatment of in-stent restenosis, with clinical and economic impacts on health-care systems.

The evolution of stent design, which has produced increasingly safer and easier-to-use devices, extended the use of PTCA, allowing approach to multivessels disease as an alternative to coronary bypass surgery. However, the increasing volume of cardiac surgery in the past few years[4] suggests that the widespread use of coronary interventions has been extended more to medical than surgical patients Therefore in-stent restenosis can be expected to increase as coronary stenting becomes more frequent, being used in a wider spectrum of patients and types of coronary lesions.

Mechanisms of restenosis

Restenosis is the reduction of the luminal size due to loss of gain in lumen size after intravascular interventional procedure. Several cellular and molecular events occur sequentially after a vascular injury.[2,5] The initial response of the elastic fibres of the vascular wall to overstretching by balloon catheter is elastic recoil, responsible for the loss of gain, which characterises the early phase of restenosis. The endothelial denudation and the exposure of subintimal components cause platelet adherence and aggregation, fibrinogen binding, and thrombus formation. Thrombus formation can also act as a scaffold into which vascular smooth-muscle cells can migrate, synthesise matrix and collagen, and reorganise the thrombus, providing the substrate for neointimal formation. Activated platelets release several mitogens and chemotactic factors, which stimulate smooth-muscle-cell migration and proliferation into the injury site. Inflammatory mediators and cellular elements contribute to trigger a complex array of events that modulate matrix production and cellular proliferation. Finally, remodelling, a gradual dynamic process mediated by adventitial myofibroblasts that leads to a change in vessel size by constrictive remodelling without an overall change in tissue volume, contributes to the loss of lumen at later times. Stenting reduces elastic recoil and negative remodelling, the mechanical component of restenosis, but also stimulates the cellular mechanisms yielding to in-stent restenosis.

By contrast with balloon angioplasty, restenosis after stenting is due mostly to neointimal formation. The bulk of in-stent restenosis consists of extracellular matrix, proteoglycans, and collagen, with only 11% cells. Greater



Drug-eluting stents: mitosis inhibition
VSCM=vascular smooth muscle cell, MMp=matrix metalloproteinase, FKBP=tacrolimus binding protein, mTOR=mammalian target of sirolimus.

neointimal proliferation is associated with deeper medial penetration of stent struts, contradicting the idea that in percutaneous coronary interventions a larger lumen achieved by angioplasty diminishes the rate of restenosis. Moreover arterial medial disruption and lipid-core penetration by stent struts is associated with greater numbers of inflammatory cells by contrast with strut in contact with fibrous plaque, highlighting the role of inflammation in restenosis and its relation with the morphology of the atherosclerotic plaque.

Preventing restenosis

Much research into many mechanical devices and drugs has been done to prevent restenosis, providing the rationale for an enormous number of clinical trials, but none have been proven to be effective.[4,7,9,11] Many different biological mechanisms contribute to restenosis and drugs that target only one pathway for a restricted period may have limited value in a multifactorial process. Experience with systemically administered drugs, such as antiplatelet agents, anticoagulants, calcium-channel blockers, angiotensin-converting-enzyme inhibitors, cholesterol-lowering agents, and antioxidants, has proven almost universally negative. These agents were previously tested in animal models and found to be beneficial. The lack of efficacy in human studies may be in part due to insufficient concentration of drug at the injury site or lack of chronic dosing. In general, although animal models provide new insight into the mechanism of restenosis, biological and mechanical differences meant that therapeutic success of anti-restenotic therapies was not achieved in human beings. The recent introduction of intracoronary radiation has emerged as a promising modality to attenuate the intimal hyperplastic reaction.[12,14] Despite the lack of benefit for preventing restenosis in de-novo lesions, brachytherapy was effective in reducing recurrent restenosis. However, larger studies and long-term follow-up showed alarming long-term sequelae such as edge restenosis and late thrombosis, providing some concerns about the potential lifelong effects of such a cytotoxic approach.

Drug-eluting stents: evolution or revolution?

The potential usefulness of immunosuppressive agents in the treatment of restenosis arises from parallels between tumour cell growth and the benign tissue proliferation which characterises intimal hyperplasia (figure). Avoiding systemic toxicity, stent-based local drug-release at the site of vascular injury via a polymeric-coated stent is an attractive therapeutic method to achieve an effective local concentration of drug for a designed period. The safety and efficacy of such an approach critically depends on the delicate combination of drug, polymer, and kinetics of release.[15] A drug-eluting stent is a device releasing into the bloodstream single or multiple bioactive agents that can deposit in or affect tissues adjacent to the stent. Drug can be simply linked to the stent surface, embedded and released from within polymer materials, or surrounded by and released through a carrier. The carrier can coat (strut-adherent) or span (strut-spanning) the stent struts

Several antiproliferative agents with different stent design are under investigation for their safety and efficacy in the treatment of coronary lesions (see webtable,

http://image.thelancet.com/extras/02cmt261webtable.pdf). Sirolimus is a natural macrocyclic lactone with potent immunosuppressive and anitimitotic action, which was approved in 1999 as an antirejection drug in renal transplant recipients. Sirolimus blocks cell-cycle progression and expression of inflammatory cytokines, thus inhibiting cellular proliferation. With the hypothesis that the immunosuppressive properties of sirolimus might inhibit neointimal proliferation, a drug eluting stent was made by coating the stent with a mixture of synthetic polymers blended with sirolimus. A second layer of drug-free polymers allows gradual drug release in a controlled concentration and time, over 30 days. Preliminary studies on animal models indicated a substantial reduction of intimal hyperplasia without adverse effect.[16]

Marie Claude Morice and colleagues[17] report the first randomised double-blind trial (RAVEL study) comparing the coronary stent coated with sirolimus with a standard uncoated stent. The trial included 238 patients with single coronary lesions at 19 different medical centres. Patients with complex coronary lesions were excluded. The angiographic rate of restenosis at 6 months was 26·6% in the standard-stent group and 0% in the drug-stent group. There were no reported cases of subacute thrombosis. At 2 years' follow-up in a subgroup of patients[18] the beneficial impact of neointimal growth inhibition was persistent. These data are indeed impressive and offer substantial hope for the elimination of in-stent intimal hyperplasia.

Sirolimus may be the first successful attempt at a drug-eluting stent but many other drugs (other immunosuppressants, antineoplastic agents, vascular endothelial growth factor, 17-β-oestradiol) are under evaluation. However, the results with the sirolimus stent are preliminary and caution should be taken with such early data. Potential systemic or local toxic effect of the drug, especially in cases of two stents placed together and overlapping, or an inflammatory late response targeted by the polymer itself, may shift restenosis to a later time or damage the distal vascular bed. Other drug-eluting stent systems (dactinomycin , QUADS-QP2 taxane, batimatast) showed different adverse events, such as late thrombosis, delayed restenosis and aneurysm formation.[19 20] Polymer coatings by their nature typically induce inflammatory responses and fibrinoid deposits. Moreover histological studies show that the stability of polymeric material may degrade over time, bringing the risk of delayed intimal hyperplasia. Therefore the perfect carrier (ie, biodegradable polymers, multilayered polymers for multiple-drug release, antigen-antibody coatings to capture endothelial cells) is still being searched for. Most importantly, to be applicable in clinical practice, drug-eluting stents must also be verified in patients with higher risk and complex coronary lesions.

The SIRIUS trial, a study that randomised 1100 patients to treatment with rapamycin-coated or standard stent, is investigating long-term safety in complex coronary lesions. Preliminary results show a significant reduction of in-stent (3·2% drug-stent *vs* 35·4% standard stent) and in segment restenosis (8·9% *vs* 36·3%), with no difference in adverse effects. The results are impressive: the combination of drug-eluting stent and brachytherapy may confine the indication for bypass surgery to a few limited cases.

Nevertheless, despite great enthusiasm over drug-eluting stents, after regulatory approval their average market penetration rate in Europe has only been 6%.[21] One of the most debated issues is the large increase in costs of the procedure: the price of a single device is about €2400, while an uncoated bare stent costs about €500. Complex analysis of the economic burden of restenosis (re-PTCA, hospitalisation, increase of medications and diagnostic testing, loss of productivity) versus the potential cost of drug-eluting stent in multivessels disease are debated. Over two decades after the first successful percutaneous coronary intervention[22] and after the discovery of sirolimus, a new era of vascular interventions is here. The further development of the stent as a local drug-delivery vehicle with no systemic effects looks promising. However, cost-effectiveness and long-term reliability remain to be defined.

References

1 American Heart Association. 2002 heart and stroke statistical update. Dallas: American Heart Association, 2001.
2 Bennett MR, O'Sullivan M. Mechanism of angioplasty and stent restenosis: implications for design of rational therapy. *Pharmacol Ther* 2001; **91**: 149–66.
3 Poon M, Badimon JJ, Fuster V. Overcoming restenosis with sirolimus: from alphabet soup to clinical reality. *Lancet* 2002; **359**: 619–22.
4 Serruys PW, de Jaegere P, Kiemeneij F, et al. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. *N Engl J Med* 1994; **331**: 489–95.
5 Serruys PW, Kay P, Disco C, et al. Periprocedural quantitative coronary angiography after Palmaz-Schatz stent implantation predicts the restenosis rate at six months: results of meta-analysis of the Belgian Netherlands Stent Study (BENESTENT) I; BENESTENT II AND music Trials. *J Am Coll Cardiol* 1999; **34**: 1067–74.
6 Topol EJ, Mark DB, Lincoff AM, et al. Outcomes at 1 year and economic implications of platelet glycoprotein IIb/IIIa blockade in patients undergoing coronary stenting: results from a multicenter randomized trial. *Lancet* 1999; **354**: 2019–24.
7 Moer R, Myreng Y, Molstald P, et al. Stenting In Small Coronary Arteries (SISCA) Trial: a randomized comparison between balloon angioplasty and the heparin coated beStent. *J Am Coll Cardiol* 2001; **38**: 1598–603.
8 Farb A, Weber DK, Kolodgie FD, et al. Morphological predictors of restenosis after coronary stenting in humans. *Circulation* 2002: **105**: 2974–80.
9 Vom Dahl J, Dietz U, Haager P, et al. Rotational atherectomy does not reduce recurrent in-stent restenosis: results of the Angioplasty Versus Rotational Atherectomy for Treatment of Diffuse In-Stent Restenosis Trial (ARTIST). *Circulation* 2002; **105**: 583–88.
10 The ERASER Investigators. Acute platelet inhibition with abciximab does not reduce in-stent restenosis (ERASER Study). *Circulation* 1999; **100**: 799–806.
11 Holmes D, Savage M, La Blanche J-M, et al. Results of Prevention RESstenosis with Tranilast and its Outcomes (PRESTO) trial. *Circulation* 2002; **106**: 1243–50.
12 Leon BM, Teirstein PS, Moses JW, et al. Localized intracoronary gamma-radiation therapy to inhibit the recurrence of restenosis after stenting. *N Engl J Med* 2001; **344**: 250–56.
13 Waksman R, Raizner A3 Yeung AC, et al, for the INHIBIT Investigators. Use of localized intracoronary beta-radiation in treatment of in-stent restenosis: the INHIBIT randomized controlled trial. *Lancet* 2002; **359**: 551–57.
14 Grise MA, Massullo V, Jani S, et al. Five year clinical follow-up after intracoronary radiation: results of a randomized clinical trial. *Circulation* 2002; **105**: 2737–40.
15 Schwartz RS, Edelman E, for the Consensus Commitee. Drug-eluting stents in preclinical studies: recommendend evaluation from a consesus group. *Circulation* 2002; **106**: 1867–73.
16 Klugherz BD, Llanos G, Lieuallen W, et al. Twenty-eight-day efficacy and pharmacokinetics of the sirolimus eluting stent. *Coron Artery Dis* 2002; **13**: 183–88.
17 Morice MC, Serruys PW, Sousa JE, et al, for the RAVEL study group. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med* 2002; **346**: 1773–80.
18 Degertekin M, Serruys PW, Foley DP, et al. Persistent inhibition if neointimal hyperplasia after sirolimus-eluting stent implantation: long-term (up to 2 years) clinical, angiographic, and intravascular ultrasound follow-up. *Circulation* 2002; **106**: 1610–13.
19 Liistro F, Stankovic G, Di Mario C, et al. First clinical experience with a paclitaxel derivate-eluting polymer stent system implantation for in-stent restenosis. *Circulation* 2002; **105**: 1883–86.
20 Virmani R, Liistro F, Stankovic G, et al. Mechanism of late in stent restenosis after implantation of a paclitaxel derivate eluting-stent system in humans. *Circulation* 2002; **106**: 2649–51.
21 Morneault JA, Kereiakes DJ, Wright CB. Economic impact of drug eluting stents in a multiple hospital healtcare system. *Am J Cardiol* 2002; **90** (suppl 6-1): 1H–4H.
22 Gruntzig A. Transluminal dilatation of coronary artery stenosis. *Lancet* 1978; **1**: 263.

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The Effect of Blockade of the CD11/CD18 Integrin Receptor on Infarct Size in Patients With Acute Myocardial Infarction Treated With Direct Angioplasty: The Results of the HALT-MI Study

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OBJECTIVES The purpose of this study was to determine whether Hu23F2G (LeukoArrest), an antibody to the CD11/CD18 integrin receptors, would reduce infarct size in patients undergoing primary angioplasty for an acute myocardial infarction.

BACKGROUND Reperfusion injury in acute myocardial infarction has been shown experimentally to be related to neutrophil accumulation. Inhibitors of the CD11/CD18 or CD18 integrin receptors have been shown to reduce infarct size in experimental models.

METHODS Patients within 6 h of onset of chest pain with ST-segment elevation were randomized to receive either 0.3 mg/kg or 1.0 mg/kg of Hu23F2G or placebo just before angioplasty of occluded arteries (Thrombolysis in Myocardial Infarction TIMI flow grade 0 or 1). The primary end point was infarct size as measured by sestamibi single-photon emission computed tomography (SPECT) scan five to nine days later.

RESULTS Four-hundred and twenty patients were enrolled and received a placebo or the study drug. The groups did not differ in baseline or angiographic characteristics or angioplasty results. Infarct size was 16%, 17.2% and 16.6%, for placebo, 0.3 mg/kg and 1.0 mg/kg, respectively, of the left ventricle (p = NS). No differences were evident in those patients with anterior myocardial infarction or those presenting within 2 h of onset of chest pain. Corrected TIMI frame count was also not different between groups. Clinical events at 30 days were very low, with a mortality of 0.8%, 1.4% and 3.3%, respectively. The drug was well tolerated, with a slight increase in minor infections in the high dose group.

CONCLUSIONS The results of this multicenter, double-blind, placebo-controlled, randomized clinical trial demonstrated that an antibody to CD11/CD18 leukocyte integrin receptor did not reduce infarct size in patients who underwent primary angioplasty. (J Am Coll Cardiol 2002;40: 1199–204) © 2002 by the American College of Cardiology Foundation

Reperfusion therapy reduces infarct size, but reperfusion injury may limit its benefits (1–8). Experimental studies have demonstrated that the inhibition of the CD11/CD18 leukocyte integrin receptor can result in a significant reduction of infarct size (9–14). In addition, inhibition can improve endothelial function, coronary blood flow and left ventricular hemodynamics. Hu23F2G (LeukArrest) is a humanized antibody directed against all isoforms of the CD11/CD18 integrin receptor (15). It has been shown to inhibit the attachment and transmigration of neutrophils, as well as adhesion-mediated release of oxygen-free radicals in vitro (oral communication, ICOS Corp., October 1997).

This multicenter, randomized, double-blind study was undertaken to evaluate the safety and efficacy of Hu23F2G in reducing infarct size as determined by sestamibi single-photon emission computed tomography (SPECT) scanning in patients with ST-segment elevation acute myocardial infarction (MI) undergoing primary angioplasty.

METHODS

This study was conducted at 54 study centers in the U.S. that were capable of performing primary angioplasty (see complete Appendix 1 online at www.cardiosource.com/jacc.html). Patients were eligible for participation if they were between the ages of 18 and 85, had chest pain or other typical signs or symptoms of acute MI of less than 6 h in duration and were candidates for primary angioplasty. For anterior MI, the electrocardiogram (ECG) entry criteria were ST-segment elevation of at least 2 mm in at least two contiguous precordial leads or new left bundle branch block. For inferior MI, the ECG entry criteria were at least 1-mm ST-segment elevation in two or more inferior limb leads (II, III, AVF), with reciprocal ST depression of at least 0.5 mm in two or more precordial leads. Patients were excluded if they had a history and ECG evidence of previous Q-wave MI or an ECG pattern that made the diagnosis of MI difficult. Other exclusion criteria were cardiogenic shock,

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Abbreviations and Acronyms
CK-MB = creatnine kinase-MB fraction (primarily in cardiac muscle)
ECG = electrocardiogram
MI = myocardial infarction
SPECT = single-photon emission tomography
TIMI = Thrombolysis In Myocardial Infarction

treatment with thrombolytic therapy before study drug administration, a baseline serum creatinine of >2 mg/dl, evidence of an ongoing bacterial infection, pregnancy and the presence of other serious medical conditions.

After informed consent, patients were randomized into the study and then immediately taken to the cardiac catheterization laboratory, where coronary angiography was performed and Thrombolysis In Myocardial Infarction (TIMI) flow grade determined. The infarct-related artery was initially injected to determine TIMI flow just before the administration of the study drug (16). Subsequently, multiple views of both coronary arteries were obtained after intracoronary nitroglycerin administration. Care was taken to ensure that the entire vessel and catheter was visualized to calculate TIMI frame count. If the infarct artery showed TIMI 0 or 1 flow grade, the patient was enrolled into the study. Patients who showed TIMI 2 or 3 flow grade did not receive the study drug but had complete screening data obtained. The study drug was administered as a weight-adjusted, intravenous bolus over 1 to 2 min. Patients were randomized to receive either 0.3 mg/kg or 1 mg/kg of Hu23F2G IV or placebo. Previous studies in normal volunteers have shown that these doses resulted in an 80% saturation of the CD11/CD18 receptor for 12 to 24 h. These doses, however, were lower than those shown to be neuroprotective in experimental studies (that is, 4 mg/kg). All patients received aspirin and heparin that was administered by intravenous infusion to maintain the activated clotting time between 250 and 300 s. Angiography was performed using standardized techniques of angioplasty with initial passage of a guidewire and balloon dilation. The use of glycoprotein IIb/IIIa antagonists was permitted, as was the use of coronary stents or other devices, at the discretion of the operator. Only the infarct-related artery was treated.

After the procedure, the sheaths were removed. The patients were then treated in the Coronary Care Unit. The institution of angiotensin-converting enzyme inhibitors, beta-blockers and platelet antagonists such as ticlopidine or clopidogrel were used at the discretion of the investigator.

Five to nine days after the administration of the study drug, a technetium-99m sestamibi SPECT image was obtained. Patients were discharged from the hospital, and they returned for an outpatient visit at 30 days for clinical assessment and determination of adverse events. At 6 months, the patients were contacted by telephone to determine their vital status and the need for rehospitalization and follow-up procedures.

Nuclear imaging technique. The nuclear core laboratory (Mayo Clinic Foundation, Rochester, Minnesota) initially validated adequate image quality at each site using a cardiac phantom and a quality control test (17). Those centers with single-headed cameras were required to use either a step-and-shoot mode or a continuous mode in circular orbit. Thirty images were required in a 64- × 64-word mode matrix over a 180° arc, beginning at 45° right anterior oblique and ending in the left posterior oblique position, with an image time of greater than 40 s. For multihead SPECT systems, acquisition was performed over 360° every 6°. Data were stored in 64- × 64-word mode matrix with an image time of greater than 30 s. Raw unprocessed data, the most recent 30 M count flood images and the most recent center of rotation study were forwarded to the core laboratory, which was blinded to treatment assignment. Measurement of infarct size has been described and validated previously (17–19). Quantitation of the extent of left ventricle with absent perfusion was performed using a five-slice technique, with short axis slices obtained every 6 mm. Quantitation was performed using threshold techniques and standard geometrical formulas. Infarct size was expressed as the percent of left ventricle with a perfusion defect.

Core angiographic laboratory. The core angiographic laboratory (University of Washington, Seattle) blindly reviewed all angiograms. The TIMI flow and corrected TIMI frame counts were determined as previously defined (16). Quantification of the coronary stenoses was performed by two readers who had no knowledge of the treatment assignment. Collaterals to the infarct-related artery were graded as present or absent. If present, the degree of perfusion was graded as full if the entire vessel was visualized and partial if only a portion of the vessel was visualized.

Safety monitoring. A safety summary was faxed to the coordinating center on day 9 or at hospital discharge. An Independent Data and Safety Monitoring Committee periodically reviewed the status of the trial.

Statistical analysis. The sample size was calculated to detect a reduction in infarct size of 8.5% of the left ventricle in patients with anterior MI and a reduction of 3.3% in patients with inferior MI. Using the variability reported elsewhere for similar patients (18,19), 112 patients per treatment group were required for two-sided p value of <0.05 at 80% power. Assuming the loss of patients during the study, 140 patients per treatment group were randomized.

The primary analyses were performed using the intention to treat the population; the results were also analyzed according to the treatment they actually received. Analyses of variance was used for comparing baseline characteristics across groups for continuous variables. The Fisher exact test was used for comparing treatment groups for categorical variables.

For the primary efficacy end point, myocardial infarct

Table 1. HALT-MI: Demographics and Baseline Characteristics

	Hu23F2G		
	0.3 mg/kg (n = 128)	1 mg/kg (n = 139)	Placebo (n = 153)
Mean age (yrs)	60.4	59.8	60.2
Percent male	73	74	69
Caucasian (%)	89	83	85
Infarct location			
Anterior (%)	33	36	34
Inferior/other (%)	67	64	66
Mean time chest pain to hospital (h)	1.66	1.70	1.71
Mean time chest pain to balloon (h)	3.9	3.7	3.7
Medical history			
Prior MI (%)	13	12	13
Hypertension (%)	50	48	48
Hypercholesterolemia (%)	44	35	44
Cigarette smoker (%)	67	78	67
Prior stent placement (%)	3	3	5
Prior PTCA/atherectomy (%)	9	7	12
Prior CABG (%)	9	4	3
Diabetes (%)	20	17	17

CABG = coronary artery bypass grafting; MI = myocardial infarction; PTCA = percutaneous transluminal coronary angioplasty.

size, determined by technetium-99m sestamibi SPECT imaging, was compared using analysis of co-variance. The following variables were used as covariates: area under the curve for the first 6 h of creatine kinase-MB fraction (CK-MB), time between initial symptoms and first balloon deflation, age, gender, pretreatment TIMI flow and infarct location. The primary analysis used imputed values for missing data. Patients who died before imaging (n = 8) were assigned the largest infarct size measured for that infarct location (anterior or other) for each patient. For patients who were alive but who did not obtain a follow-up SPECT image (n = 22), the median value measured for each infarct location was used. A Cox proportional hazards regression model was used for the survival analysis.

RESULTS

Patient population. Six-hundred and thirty-seven patients were randomized and 420 patients with TIMI flow grade 0 or 1 flow were enrolled. One-hundred and sixty-nine patients of 214 did not receive study drug because of their TIMI 2 or 3 flow grade at initial angiography. One hundred and twenty-eight received 0.3 mg/kg of LeukoArrest, 139 patients received 1 mg/kg of LeukoArrest and 157 patients received placebo. The follow-up was complete at 30 days in 99% of patients. Fourteen patients (seven from the placebo group) received a different therapy than initially assigned.

Baseline characteristics. The baseline demographic and clinical characteristics of the 420 patients enrolled were not different between groups receiving the study drug versus the placebo (Table 1). Medication use during hospitalization was not different between groups. The angiographic findings showed the infarct artery to be the left anterior descending in 33% to 36% (Table 2). TIMI 0 or 1 flow grade was confirmed by subsequent core angiographic lab assessment in 96% to 98% of patients. Collateral vessels were present to the infarct related artery in 32% to 40% of patients. Full collateralization was present in 29% to 37% percent of those who had collaterals.

Table 2. HALT-MI: Angiographic and Angioplasty Results

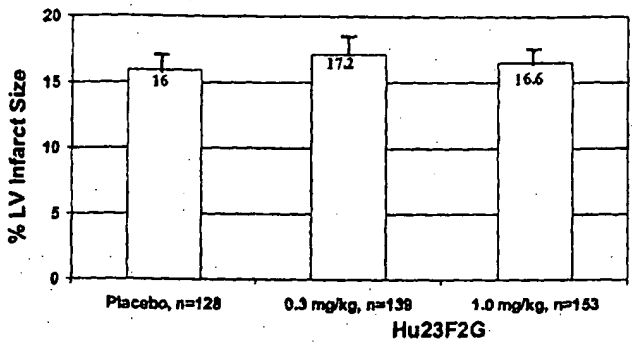
	Hu23F2G		
	0.3 mg/kg (n = 128)	1 mg/kg (n = 139)	Placebo (n = 153)
Infarct vessel			
LAD (%)	34	36	34
Circumflex (%)	14	13	9
RCA (%)	52	51	57
TIMI flow pre-PTCA (%)			
0-1	98	96	96
2	2	4	3
Collaterals to IRA (%)	32	42	41
Angiographic success	88	86	89
Percent residual stenosis	8	6	8
TIMI flow-post (%)			
0-1	6	6	4
2	9	4	8
3	81	86	86
Corrected TIMI frame count	21.3	23.3	22.3

IRA = infarct-related artery; LAD = left anterior descending; MI = myocardial infarction; PTCA = percutaneous transluminal coronary angioplasty; TIMI = Thrombolysis In Myocardial Infarction.

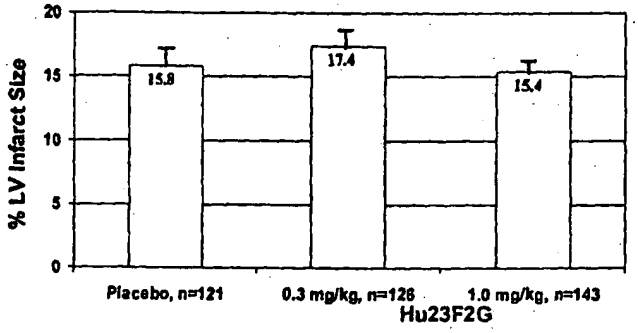
Angioplasty results. A successful procedure, defined as less than a 50% residual stenosis without death or emergency bypass surgery, was seen in 86% to 89% of each group (Table 2). Stents were placed in 85% of patients and abciximab was used in 65% to 73% of patients. The residual stenosis was less than 9% in all three groups; TIMI 3 flow grade was established in 81% to 86% of patients. None of the differences among the three groups were statistically significant.

Infarct size. The final infarct size for the intention-to-treat population, with and without imputation, is shown in Figure 1A and 1B. Including imputed values for missing data, infarct size was 16%, 17.2% and 16.6% of the left ventricle for placebo, 0.3 mg/kg and 1 mg/kg, respectively (p = 0.796). The observed data (without imputation) on 390 patients (92.9% of the study group) gave similar results.

The subgroup of patients with anterior MI had significantly greater infarct size (averaging 25%), but again no differences were noted among the three groups. Likewise, when patients were subdivided by the time from the onset of chest pain to balloon deflation or by the presence of collaterals, there were no differences. Infarct size, measured by CK-MB area under the curve for 24 h, was not different among the three groups (175%, 175% and 184% per hour for placebo, 0.3 mg/kg and 1 mg/kg, respectively). Microvascular flow, assessed by corrected TIMI frame counts, were also not different among the groups (21.3%, 23.2% and 22.3%, respectively).



A Imputed Values, Mean ± SE



B Observed Data, Mean ± SE

Figure 1. (A and B) HALT-MI infarct size by single-photon emission computed tomography, imputed values and observed data. LV = left ventricular.

Clinical events at 30 days. The incidence of major clinical events was very low, with only eight deaths in the study population, for an overall 30-day mortality of 1.9% (Table 3). Although there were fewer deaths and reinfarctions in the low dose group (0.3 mg/kg) and survival at 30 days tended to be better in the treatment groups, there was no significant statistical difference ($p = 0.20$), given the low number of events. When a composite end point using death, new MI, rehospitalization for heart failure and infarct size by SPECT scanning were used, no differences between groups could be discerned.

Adverse events. Major infections occurred in 5% to 10% of patients, with minor infections in 2% to 9%. The most frequent infection was urinary tract infection, which occurred in 6.3%, 9.4% and 2.6% of patients in the 0.3 mg/kg, 1 mg/kg and placebo group, respectively. Infections were more common in the active treatment group but were easily managed and caused no serious adverse events.

Table 3. HALT-MI: Clinical and Adverse Events at 30 Days

Event	Hu23F2G 0.3 mg/kg (n = 128)	Hu23F2G 1 mg/kg (n = 139)	Placebo (n = 153)
Clinical events			
Death (%)	0.8	1.4	3.3
Reinfarction (%)	0.8	2.9	3.9
New CHF (%)	1.6	1.4	3.3
Revascularization (%)	12.5	17.3	10.5
Rehospitalization (%)	8.6	10.1	13
Adverse events			
Major infection (%)	6	10	5
Minor infection (%)	5	9	2
Bleeding (%)	16	17	15

CHF = congestive heart failure.

DISCUSSION

The results of this multicenter, randomized, double-blind, placebo-controlled, parallel group study demonstrated that Hu23F2G, an antibody directed against the leukocyte CD11/CD18 integrin receptor, did not significantly reduce infarct size, as determined by Tc-99m sestamibi SPECT scanning five to nine days after primary angioplasty. The drug was well tolerated in this study population, although infections were slightly more common in the high dose group.

Neutrophils in reperfusion injury. Reperfusion of ischemic myocardium, particularly with oxygenated perfusate, has been shown to contribute to further tissue damage and has been called reperfusion injury (4–8). A number of mechanisms have been proposed for reperfusion injury, including an excess release of oxygen free radicals. Inflammation during acute MI has been recognized for more than 60 years (20). Experimental and clinical studies have demonstrated an increase in neutrophil accumulation in the infarct zone upon reperfusion, with an increase in cytokine release and expression of the CD11/CD18 integrin receptors on white cells (21–24) before, but particularly after, reperfusion, with the plugging of small arterioles and capillaries (25). Clinical estimation of microvascular flow after reperfusion using the rate of resolution of ST-segment changes, corrected TIMI frame count, or the density of myocardial contrast has identified patients at higher risk for subsequent cardiovascular events (26–28).

Inhibitors of the CD11/CD18 integrin receptor. Antibodies to either all or one of the four isoforms of the CD11/CD18 integrin receptor have been shown to reduce infarct size, improve coronary blood flow, improve left ventricular function and decrease neutrophil infiltration (9–14).

Hu23F2G (LeukArrest), a humanized antibody against all four leukocyte integrins, has been shown to inhibit leukocyte attachment and transmigration (15). In normal volunteers, a greater than 80% saturation of the CD11/CD18 neutrophil receptor inhibited chemotaxis of granulocytes using a skin chamber (personal communication, ICOS Corp.). In a pilot study of 60 patients undergoing primary angioplasty with acute MI, using the same doses as used in this study, an 80% saturation of the receptor was demonstrated from 24 to 48 h (29). Given the negative results of this study, it is possible that this degree of saturation of neutrophil CD11/CD18 receptors is insufficient to reduce infarct size. It is possible that the use of GP IIb/IIIa agents in this study may have influenced the results. Some studies have suggested that abciximab inhibits the

MAC-1 receptor, and this may have similar effects to LeukArrest (30). The results of the Limitation of Myocardial Injury following Thrombolysis in Acute Myocardial Infarction (LIMIT AMI) trial, which used an antibody against the CD18 receptor in patients with acute MI receiving thrombolysis, also failed to show a significant difference in infarct size as measured by Tc-99m sestamibi or TIMI flow (31).

Other agents to reduce reperfusion injury. Clinical trials of many other agents directed toward reducing reperfusion injury have been similarly disappointing. Prostacyclin, fluosol, magnesium, poloxmer 188 (rheothrx) and tremetazidine have also failed to reduce infarct size in randomized clinical trials (32–34) (M. Marzilli, unpublished data). In contrast, adenosine has shown promise (35,36). Adenosine is a potent vasodilator that also has cardioprotective properties, most likely through the replenishment of high-energy phosphate stores, inhibition of oxygen-free radicals, inhibition of neutrophils and improvement of microvascular flow and ischemic preconditioning (36). The Acute Myocardial Infarction Study of Adenosine (AMISTAD) study showed a 33% reduction in infarct size, particularly in patients with anterior MI (15% vs. 45.5%), when given to patients at the time of thrombolysis (35). In contrast to the AMISTAD study, this study had a very low incidence of complications and death. This could be due to the effectiveness of primary angioplasty in the treatment of acute MI and/or other variables, such as patient selection. Although the sites chosen to participate in the study had considerable clinical experience in primary angioplasty, the majority were not academic medical centers. Therefore, the results are likely to reflect the contemporary practice of primary angioplasty in patients with their first ST elevation infarction.

Study limitations. This study was sized to demonstrate a decrease in infarct size of 8.5% of the left ventricle in anterior MI and a decrease of 3.2% in inferior MI. The design assumptions were fairly well satisfied, with an 80% power. However, the study was underpowered to detect a smaller degree of benefit. Because the area at risk was not measured, it is possible that measurement of myocardial salvage would have been a more sensitive measure than final infarct size. Previous studies, however, have shown a close relationship between the two (18), and the logistical issues led to the decision not to determine myocardial salvage. The dose was chosen to insure at least 80% granulocyte receptor saturation for 12 to 24 h, which in Phase 1 studies in normal volunteers resulted in a significant inhibition of chemotaxis, using a skin window study. However, the dose of drugs and the timing of administration may not have been sufficient to inhibit the intense neutrophil accumulation known to occur during acute MI. Experimental studies have shown that higher doses are necessary to demonstrate neuroprotection. Although infarct size has been established as a surrogate end point for acute MI trials (19), it is possible that the therapy might have clinical benefits independent of infarct size, which might have been detected in a much larger trial using clinical end points.

Conclusions. The results of the multicenter, randomized trial demonstrate that an antibody to the CD11/CD18 leukocyte integrin receptor did not reduce infarct size in patients undergoing primary angioplasty. Ongoing studies of other leukocyte integrin receptor blockers will help to better define the role of neutrophil inhibition in reducing infarct size.

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REFERENCES

1. American Heart Association. 2000 Heart and Stroke Statistical Update. Dallas, TX: American Heart Association, 2000;1–39.
2. Fibrinolytic Therapy Trialists' (FTT) Collaborative Group. Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomized trials of more than 1,000 patients. Lancet 1994;343:311–22.
3. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword. J Clin Invest 1985;76:1713–19.
4. Hearse D, Bolli R. Reperfusion induced injury: manifestations, mechanisms and clinical relevance. Cardiovasc Res 1992;26:101–8.
5. Maxwell SRJ, Lip GYH. Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options. Int J Cardiol 1997;58:95–117.
6. Hansen PR. Role of neutrophils in myocardial ischemia and reperfusion. Circulation 1995;91:1872–85.
7. Neumann FJ, Ott I, Gawaz M, et al. Cardiac release of cytokines and inflammatory responses in acute myocardial infarction. Circulation 1995;92:748–55.
8. Grisham MB, Granger DN, Lefer DJ. Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. Free Radical Biol Med 1998;25:404–33.
9. Lefer DJ, Shadelya SML, Serrano CV, Becker LC, Kuppusamy P, Zweier JL. Cardioprotective actions of a monoclonal antibody against CD-18 in myocardial ischemia-reperfusion injury. Circulation 1993; 88:1779–87.
10. Tanaka M, Brook SE, Richard VJ, et al. Effect of anti-CD18 antibody on myocardial neutrophil accumulation and infarct size after ischemia and reperfusion in dogs. Circulation 1993;87:526–35.
11. Aversano T, Zhou W, Nedelman M, Nadada M, Weisman H. A chimeric IgG4 monoclonal antibody directed against CD18 reduces infarct size in a primate model of myocardial ischemia and reperfusion. J AM Coll Cardiol 1995;25:781–8.
12. Arai M, Lefer DJ, So T, DePaula A, Aversano T, Becker LC. An anti-CD18 antibody limits infarct size and preserves left ventricular function in dogs with ischemia and 48-hour reperfusion. J Am Coll Cardiol 1996;27:1278–85.
13. Horwitz LD, Kaufman D, Kong Y. An antibody to leukocyte integrins attenuates coronary vascular injury due to ischemia and reperfusion in dogs. Am J Physiol 1997;272:H618–24.
14. Palazzo AJ, Jones SP, Giron WG, Anderson DC, Granger DN, Lefer DJ. Myocardial ischemia-reperfusion injury in CD18 and ICAM-1 deficient mice. Am J Physiol 1998;275:H2300–7.
15. Yenari MA, Kunis D, Sun GH, et al. Hu23F2G, an antibody recognizing the leukocyte CD11/CD18 integrin, reduces injury in a rabbit model of transient focal cerebral ischemia. Exp Neurol 1998; 153:223–33.
16. Gibson CM, Cannon CP, Daley WL, et al., for the TIMI 4 Study Group. TIMI frame count. A quantitative method of assessing coronary artery flow. Circulation 1996;93:879–88.

17. O'Connor MK, Gibbons RJ, Juni JE. Quantitative myocardial SPECT for infarct sizing: feasibility of a multicenter trial evaluated using a cardiac phantom. J Nucl Med 1995;36:1130–6.
18. Gibbons RJ, Christian TF, Hopfenspirger M, et al. Myocardium at risk and infarct size after thrombolytic therapy for acute myocardial infarction: implications for the design of randomized trials of acute intervention. J Am Coll Cardiol 1994;24:616–23.
19. Gibbons RJ, Miller TD, Christian TF. Infarct size measured by single photon emission computed tomographic imaging with (99m) Tc-sestamibi: a measure of the efficacy of therapy in acute myocardial infarction. Circulation 2000;101:101–8.
20. Neumann FJ, Ott I, Gawaz M, et al. Cardiac release of cytokines and inflammatory responses in acute myocardial infarction. Circulation 1995;92:748–55.
21. Sheridan FM, Cole PG, Ramage D. Leukocyte adhesion to the coronary microvaculature during ischemia and reperfusion in an in vivo canine model. Circulation 1996;93:1734–87.
22. Kassirer M, Zeltser D, Gluzman B, et al. The appearance of L-selecting^{low} polymorphonuclear leukocytes in the circulating pool of peripheral blood during myocardial infaction correlates with neutrophilia and with the size of the infarct. Clin Cardiol 1999;22:721–6.
23. Kassirer M, Zeltser D, Prochorov V, et al. Increased expression of the CD11b/CD18 antigen on the surface of peripheral white blood cells in patients with ischemic heart disease: further evidence for smoldering inflammation in patients with atherosclerosis. Am Heart J 1999;138: 555–9.
24. Engler RL, Schmid-Schonbein GW, Pavelec RS. Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. Am J Pathol 1983;111:98–111.
25. Claeys MJ, Bosmans J, Veenstra L, Jorens P, De Raedt H, Vrints CJ. Determinants and prognostic implications of persistent ST-segment elevation after primary angioplasty for acute myocardial infarction. Importance of microvascular reperfusion injury on clinical outcome. Circulation 1999;99:1972–7.
26. Gibson CM, Murphy SA, Rizzo MJ, et al., for the Thrombolysis in Myocardial Infarction (TIMI) Study Group. Relationship between TIMI frame count and clinical outcomes after thrombolytic administration. Circulation 1999;99:1945–50.
27. Gibson CM, Cannon CP, Murphy SA, et al., for the TIMI (Thrombolysis In Myocardial Infarction) Study Group. Relationship of TIMI myocardial perfusion grade to mortality after administration of thrombolytic drugs. Circulation 2000;101:125–30.
28. Wall TC, Califf RM, Blandenship J, et al. Intravenous fluosol in the treatment of acute myocardial infarction. Results of the Thrombolysis and Angioplasty in Myocardial Infarction 9 Trial. Circulation 1994; 90:114–20.
29. Rusnak JM, Kopecky SL, Clements IP, et al., for the FESTIVAL Investigators. An anti-CD11/CD18 monoclonal antibody in patients with acute myocardial infarction having percutaneous transluminal coronary angioplasty (The FESTIVAL Study). Am J Cardiol 2001; 88:482–7.
30. Bronfman S. Potential non-glycoprotein IIb/IIIa effects of abciximab. Am Heart J 1999;138:S1–5.
31. Baran K, Nguyen M, McKendall GR, et al., for the LIMIT AMI Investigators. Double-blind, randomized trial of an anti-CD18 antibody in conjunction with recombinant tissue plasminogen activator for acute myocardial infarction. Limitation of Myocardial Injury following Thrombolysis in Acute Myocardial Infarction (LIMIT AMI) Study. Circulation 2001;104:2778–85.
32. ISIS-4 (Fourth International Study of Infarct Survival) Collaborative Group. ISIS-4: a randomized factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulfate in 58,050 patients with suspected acute myocardial infarction. Lancet 1995;345: 669–85.
33. Collaborative Organization for RheothRx Evaluation (CORE). Effects of RheothRx on mortality, morbidity, left ventricular function, and infarct size in patients with acute myocardial infarction. Circulation 1997;96:192–201.
34. EMIP-FR Pilot Study Group. Free radicals, reperfusion and myocardial infarction therapy: European Myocardial Infarction Project—Free Radicals Pilot Study. Eur Heart J 1993;14 Suppl G:48–51.
35. Mahaffey KW, Puma JA, Barbagelata A, et al, for the AMISTAD Investigators. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial. J Am Coll Cardiol 1999;34:1711–20.
36. Ely SW, Berne RM. Protective effects of adenosine in myocardial ischemia. Circulation 1992;85:893–904.

APPENDIX

For a complete list of HALT-MI Investigators, please see the October 2, 2002 issue of *JACC* at www.cardiosource.com/jacc.html.

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Review

Chapter Eight

Antithrombotic drugs

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Emerging Drugs (1999) 4:175-195

1. Summary

The haemostatic-thrombotic system (HTS) can be inhibited at dozens of different sites by hundreds of different drugs. The ideal antithrombotic is an inhibitor that downregulates the HTS without causing bleeding or other side-effects, and that can be given orally in a fixed dose. These are pharmacological criteria. Indeed, the three current therapies (heparins, oral anticoagulant therapy and aspirin) act by completely different mechanisms. It is not the mechanism of inhibition which is important but the pharmacological properties of the inhibitor; no compound has been found that inhibits the HTS and does not show an antithrombotic effect.

The HTS is a non-linear system containing a number of nested positive and negative feedback loops. At the present state of knowledge it is impossible to predict the effect of inhibition of a single reaction on the response of the complete system. For this reason one cannot predict the antithrombotic potency of a compound from its biochemical properties. It is shown that clotting tests, measurement of platelet aggregation/adhesion or tests of individual functions within the system are only of limited use. Antithrombotics therefore should be evaluated by their action on a representative function test of the complete HTS. Such a test is not routinely available at the moment. Two promising possibilities are flow chambers using non-anticoagulated blood and thrombin generation in blood or platelet rich plasma (PRP).

2. Background

Thrombosis is caused by the same mechanism that causes haemostasis. Administering antithrombotic drugs therefore requires careful manipulation of the balance between thrombosis and bleeding. For the safe use of

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antithrombotic drugs, we need a probe for the functional status of the HTS. Such a test is not routinely available at this moment. Bleeding times are hopelessly inaccurate [1] and clotting times are to be considered surrogate variables of limited use. There is no general test to indicate hypercoagulability and thrombosis risk, or to monitor the effect of antithrombotic treatment. Current management of thrombosis is like managing diabetes by the taste of the urine, or hypertension with only a pulse to feel.

This situation has an immediate influence on the search for new antithrombotic drugs. There is no easy, valid laboratory test to indicate therapeutic efficacy or optimal dosage. The research goal is defined in terms like 'doubling the activated partial thromboplastin time (aPTT)' or 'inhibition of platelet aggregation' that have, at the best, a semiquantitative relation to haemostasis and thrombosis. Further testing is in animal thrombosis models and, eventually, clinical trials.

In this article we will defend the position that it is unimportant how the function of the HTS is inhibited as long as the inhibition fulfils certain pharmacological criteria. Consequently, we will not dwell in detail on individual mechanisms of inhibition or on inhibitor design. Rather we will stress the importance of assessing HTS function as a whole.

3. Medical need

In recent years it has been firmly established that virtually every arterial occlusive event (such as myocardial or cerebral infarction) is due to the formation of a local thrombus on a damaged internal surface of the vessel. Thrombin plays an all-important role in this process [2-6]. A damaged wall exposes triggers for thrombosis, such as tissue factor (TF) present in the smooth muscle cells of neointima [7], in the adventitia and in the plaque [8-10], as well as the platelet-activating collagen surface [11]. Non-occlusive arterial thrombosis may lead to downstream occlusion if the thrombus is carried with the bloodstream. In strategic positions, such as the carotid arteries, this may have serious consequences. Arterial thrombosis is not only the consequence of atherosclerosis; through microthrombus formation it also plays a role in the development of arterial wall degeneration. Furthermore, venous thrombosis and its sequelae, ranging from sudden death due to pulmonary embolism to chronic leg-ulcers, is not a negligible source of illness. At this moment, in the Western World, arterial and venous thrombosis and atherosclerosis together are responsible for well over 50% of all mortality and serious morbidity. In a few decades this will be the case worldwide [12]. Good antithrombotic drugs, therefore, are a necessity.

3.1 Existing treatment

3.1.1 Heparins

Heparins, either unfractionated (UFH) or of the low molecular weight type (LMWH) [14], reduce the incidence of thrombosis by about 50%. They have the drawback of parenteral administration. They may cause antithrombin (AT) deficiency, thrombocytopenia, bleeding and a few more rare side-effects. The methods for laboratory control of heparin dosage, notably the aPTT, are inadequate. The question of whether adapted doses of heparin would do better than current practice remains unsolved because control methods to guide the adaptation are not available. Prolonged heparin treatment, in particular with UFH, is not favoured.

3.1.2 Oral anticoagulation

Oral anticoagulation (OAC) is maintained with coumarin congeners, i.e., anti-vitamin K drugs. They inhibit the oxidation reduction cycle of vitamin K in the liver, required for the normal γ-carboxylation of the clotting Factors II, VII, IX and X, protein C and protein S. There are large variations in individual need and many drug interactions (e.g., with sleeping pills and alcohol). Therefore, regular control of the level of anticoagulation is necessary. This can be readily carried out *via* determination of the thromboplastin time and expressing the result as an international normalised ratio (INR).

3.1.3 Aspirin

Aspirin is the only antiplatelet drug in general use. The effect is dose-independent between 40 and 1000 mg/day. There are few complications seen. The protective effect in venous thrombosis is not significant and in arterial thrombosis it is modest (10 - 20%). Ticlopidine and clopidogrel (Iscover™, Plavix™) are alternative possibilities. Abciximax, the antibody against the fibrinogen receptor of plasma, is used in acute coronary infarction.

4. Therapeutic class review

Heparins are glycosaminoglycans with molecular weights ranging from 1500 to 100,000 Da [14]. Those that contain a specific pentasaccharide sequence bind to AT and catalyse the irreversible inhibition of activated clotting factors by this plasma protein. The inhibitory capacity of AT towards thrombin is roughly three times that towards Factor Xa; that towards IXa and XIa is smaller and probably without practical significance. The relative activity against the different factors is not changed by heparin, with one notable exception: heparins smaller than 5400 Da will not cause inactivation of thrombin, but do catalyse that of Factor Xa. Inhibition of Factor Xa is an inefficient way to inhibit thrombin formation. LMWHs contain a fair amount of molecules of < 5400 Da, whereas UFH does not; therefore LMWHs have a higher anti-Xa activity than UFH. The difference is small in the presence of Ca^{++} ions (i.e., *in vivo*) but appears much larger in the usual *in vitro* test, where Ca^{++} is absent [15]. A high anti-Xa activity has been the guideline in searching for efficient LMWHs [14]. Despite the inefficiency of Factor Xa inhibition this has led to useful products, because preparations with a high anti-Xa activity also contain a large proportion of material in the 5400 – 9000 Da range. Such material is an efficient anticoagulant because of its persistent AT activity, together with a high bioavailability after sc. injection [16,17]. It has also been surmised that the very large molecular weight components present in UFH, but not in LMWH, have specific haemorrhagic properties [18]. In retrospect, the anti-Xa activity must be considered largely an artefact; LMWHs are good drugs developed on the basis of a mistaken concept.

AT only partially inhibits clot-bound thrombin, if at all. Heparin co-factor II (HCII) [19] is capable of inhibiting this thrombin, but only in the presence of relatively high concentrations of acid polysaccharides (dermatan sulfate, pentosan polyphosphate, lactobionic acid, etc.) [20-24]. Heparin can also activate HCII, but at doses much higher than the normal therapeutic and preventive ones.

4.1 Direct thrombin inhibitors

The prototype of direct thrombin inhibitors is hirudin [25-28], which has proven its value in experimental thrombosis as well as in clinical trials. Indeed direct thrombin inhibition is a feasible route to obtain antithrombotic action [6,29-33]. Modern techniques of

combinatorial chemistry can construct large peptide derivative libraries and facilitate the search for specific inhibitors [34,35]. There is also continuous interest in natural inhibitors from haematophagous animals and other sources [36-40], CVS1123 [41], inogatran (HA-31427) [42-44], melagatran (H-31968) [42-47] and napsagatran (Ro-46-6240) [48], and a large variety of others [49-65]. Some have been tested in the clinical situation. Most of them are administered parenterally, but oral administration is feasible [66]. The overall impression is that, like hirudin, they can replace heparin, without showing obvious advantages except their use in AT deficiency and their ability to inactivate clot-bound thrombin [44,49,67,68].

There is an essential difference between irreversible thrombin inhibitors (such as hirudin) and reversible ones (such as argatroban [Novastan™]). The irreversible ones bind with 1:1 stoichiometry to thrombin. If drug administration is stopped, the inactivated thrombin molecules remain inhibited. Serious bleeding problems are to be expected as soon as the concentration of an irreversible inhibitor approaches the level of prothrombin in plasma (~2μM), so that all the thrombin that can be formed is immediately inhibited. Reversible inhibitors bind a fraction of the available thrombin; the proportion inhibited is dependent upon the inhibition constant (K_i = the concentration at which half-maximal inhibition is observed) and upon the concentration of the inhibitor. If the concentration of the inhibitor decreases, the inhibition is relieved. Reversible inhibitors probably act because they diminish thrombin concentration at the site of thrombosis, where thrombin formation is triggered. They will enter into competition with the natural substrates of thrombin (procoagulants: e.g., fibrinogen; Factors V, VIII, XI; platelets, as well as anticoagulants: e.g., thrombomodulin). All reversibly inhibited thrombin molecules will be eventually released and taken over by natural, irreversible inhibitors (e.g., AT). So, irreversible inhibitors arrest thrombin action and reversible ones dampen it. Both mechanisms seem to lead to antithrombotic activities. It is unknown to the authors whether there are fundamental differences in haemorrhagic properties.

4.2 Direct inhibitors of other clotting enzymes

A large number of natural and synthetic inhibitors of Factor Xa has been described [35,69-86]. The original idea behind their development was that it is more efficient to inhibit thrombin production ('closing the tap') than thrombin itself ('mopping the floor'). This view, based upon the early concepts of the clotting cascade as a linear mechanism, became untenable as soon as it was realised how important thrombin-mediated feedback reactions are and how important Factor Xa is for the inhibition of the TF Factor VIIa complex (TF-VIIa) *via* tissue factor pathway inhibitor (TFPI). Also, Factor Xa has to be inhibited by 90% in order to inhibit prothrombin conversion by about 50%.

Inhibition of the first step in thrombin generation, i.e., at the level of Factor VIIa and TF, is also possible [87-89]. An interesting concept is the inhibition of TF by the administration of recombinant, active site-inhibited Factor VIIa [90-92], or by peptides that mimic part of this molecule [93].

All VIIa and Xa inhibitors tested appear capable of inhibiting experimental thrombosis (e.g., [94-96]). They have not yet been used in the clinic, and it is not established whether they show specific advantages. However, an important finding has recently been made that shows that circulating TF plays a role in the formation of the thrombus [170]. A competitive VIIa inhibitor could inhibit this thrombus formation. Theoretically, this approach towards thrombus prevention would have a broad therapeutic range since the amount of circulating

TF is much easier to inhibit than the local TF exposure after vessel damage, thus minimising bleeding risk.

The main result from research on direct inhibitors as a group is that any inhibitor of the clotting system, independent of the enzyme that it attacks or of the mode of inhibition is antithrombotic, in any thrombosis model.

4.3 Platelet inhibitors

Many excellent reviews on platelet inhibitors have been published [e.g., 97,98]. Aspirin, in all probability, acts by irreversibly inhibiting cyclooxygenase and, thereby, platelet thromboxane A_2 (TXA_2) synthesis [99]. Other non-steroidal anti-inflammatory drugs (NSAIDs) and sulfinpyrazone inhibit the same enzyme reversibly. Dipyridamole increases intraplatelet cyclic AMP (cAMP) [99-101], as do prostaglandin E1 and prostacyclin (which, due to its instability, is of limited therapeutic use) [102,103]. Stable analogues of the latter are iloprost (SH-401) [104,105] and ciprostene (U-61431F) [106]. Thromboxane synthase inhibitors and inhibitors of the thromboxane receptor are of doubtful efficiency [107]. Ketanserin, one of the group of serotonin receptor antagonists seems to do no better [108,109].

Inhibitors of the ADP receptor, such as ticlopidine [110-113] or clopidogrel [114], are better than aspirin and may be combined with it. Inhibitors of the platelet fibrinogen receptor glycoprotein (GP) IIb/IIIa have shown promising results [115-119].

Platelet inhibitors have been developed as inhibitors of adhesion or aggregation. Recently, it appeared that all those tested hitherto also inhibit thrombin generation in PRP. Aspirin, for example, causes an inhibition of about 10%, roughly equal to the reduction of arterial thrombosis that is seen in large clinical trials. Prostacyclin analogues also inhibit thrombin generation in PRP [120] (unpublished observations). Inhibition of GP IIb/IIIa, the fibrinogen receptor of the platelet membrane, reduces thrombin generation by about 50% [121,122]. Administration of these inhibitors produces the same defect as is congenitally present in Glanzmann's thrombasthenia, a disease in which a diminution of thrombin generation is observed [121,122]. Also, inhibition of von Willebrand factor (vWF), GP Ib and the vWF receptor strongly inhibits thrombin generation in PRP [123,124]. Thrombin generation in PRP is evidently also dependent upon the function of the plasmatic clotting factors and upon the interactions between plasma and platelets. This suggests that thrombin generation in PRP and/or blood might be a good screening technique for antithrombotics, independent of whether they act *via* the platelet or as anticoagulants.

5. Current research goals

The ultimate research goal is an antithrombotic that decreases the incidence of thrombosis (of any type) by more than 50%, that can be administered orally, that is immediately active, that can be given in a standard dosage without requiring laboratory control and that does not cause bleeding when overdosed. None of the available antithrombotics fulfil these criteria.

The complicated HTS lends itself to inhibition at a large number of sites, each of them a potential target for pharmacological action. The three established therapies have completely different modes of action. Multiple other modes of inhibition have been shown to work in experimental thrombosis. This proves that the desired goal can be attained in many different ways. The question of what specific target in the HTS should be attacked in

order to obtain the ideal antithrombotic is often posed. It is mostly answered *a priori* by theoretical considerations that need to be corroborated *a posteriori* by animal thrombosis experiments. The result, as stated, is that any inhibition entails antithrombotic activity. Advanced knowledge of the subsystems of the HTS may, in the long run, give the answer. This goal is not within easy reach however. At this moment, it would be more useful to conceive of a screening test that represents total HTS function, and that is simpler and less expensive than animal thrombosis models.

5.1 Laboratory models for the function of the HTS

The ideal model, like real thrombosis, should respond in a similar way to clinically effective concentrations of all antithrombotics independent of the moleculr focus of their effect. This means that clotting times in PRP (and *a fortiori* inhibition tests of individual clotting factors), platelet aggregation or adhesion tests carried out in anticoagulated PRP, are not reasonable options. In view of their widespread use, we will nevertheless discuss clotting times in more detail.

5.2 Clotting times

Blood (plasma) clots when more than 95% of the prothrombin is still to be converted [120]. Clotting times do not reflect the amount of thrombin that is still to be formed at the moment of clotting. Yet it is the amount of thrombin that determines the extent of the thrombotic reaction. Therefore, a clotting time will not reflect directly the relevant parameter. This is also seen from the fact that a different type of clotting time is required for estimating the effect of different types of anticoagulants. The thromboplastin time or 'prothrombin time' (PT), for example, indicates the depth of oral anticoagulation but is not sensitive to the effect of heparin. The aPTT is particularly sensitive to variations in the concentration and/or activity of Factor VIII and UFH, but not to LMWH and activators of HCII. Doubling the aPTT is thought to represent adequate heparin administration. This is based on very limited studies in animals [125] and man [126,127]. There is no relation between recurrent thromboembolism and the aPTT response [128]. The mechanism by which direct thrombin or Factor Xa inhibitors prolong the aPTT is significantly different from that of heparin [129]. Nevertheless 'doubling the aPTT' is also used as an indicator for therapeutic action with any type of clotting inhibitor. We conclude that clotting tests are not a valid general indicator of antithrombotic action. This does not mean that they cannot be used in special cases: the thromboplastin time is a good indicator of oral anticoagulation [130] and the Ecarin time is useful in assessing the level of direct thrombin inhibitors [131,132].

5.3 Platelet tests

Platelet aggregation and flow-chamber or other tests that are carried out on washed platelets or on PRP in which the coagulation mechanism has been inhibited, will not reflect interactions between platelets and the clotting system, and therefore are of limited use [133-135]. Experiments in which non-anticoagulated blood is passed through a flow chamber containing a thrombogenic surface are not subject to this drawback. A model system that tries to mimic the concert of thrombotic triggers and flow parameters has been developed [136-140]. A flow chamber is directly coupled to the antecubital vein of a volunteer, and flow of non-anticoagulated blood is metered by a peristaltic pump. The flow chamber contains a piece of the media of a pig aorta, cultured endothelial cells, smooth muscle cells or collagen-coated surfaces and human atherosclerotic plaques. After the experiment, the exposed material will be stained and examined for platelet and fibrin deposition. Drugs can be administered and tested for antithrombotic properties. This

model for thrombosis has proven to be a workable system that closely mimics physiologic reality under standardised conditions.

5.4 The thrombin generation curve

The thrombin generation curve (TGC), one of the oldest tools of the clotting trade, has lately become the subject of renewed interest [15,74,120,122-124,141-44]. It contains all the relevant information on the physiological function of the coagulation system. The area under the curve (the endogenous thrombin potential (ETP) [142]) represents the total enzymatic action that thrombin can develop during its lifetime in plasma. In blood and in PRP, the ETP can be measured from a TGC obtained by subsampling. If a substrate with the right kinetic properties is added to plasma, thrombin generation can be monitored continuously as the first derivative of the product-time curve. At this moment, only chromogenic substrates have been described for this use so that the method is restricted to defibrinated PRP.

We demonstrated that higher than normal ETP is seen in all blood-based thrombosis-prone states tested, either congenital (e.g., deficiency of AT, protein S or protein C, factor V Leiden, prothrombin 20210A) or acquired (e.g., from the use of contraceptive pills). In coronary infarction, months after the acute phase as well as in patients presenting with a deep vein thrombosis, the ETP is increased. In the haemophilias (deficiency of Factors VII, IX and XI) the ETP is significantly lower than normal [145].

OAC treatment (INR = 1.5 - 3.0) decreases the ETP to 20 - 40% of normal. Heparin administration in the therapeutic range has the same effect. The ETP also accurately reflects the effect of mixed OAC and heparin treatment. Platelet aggregation inhibitors diminish thrombin generation in PRP. In fact, we encountered no anticoagulant that would not decrease the ETP in PRP, and no antiplatelet agent that would not inhibit the ETP in PRP. This made us think that the ETP in PRP might be a useful model for antithrombotic action.

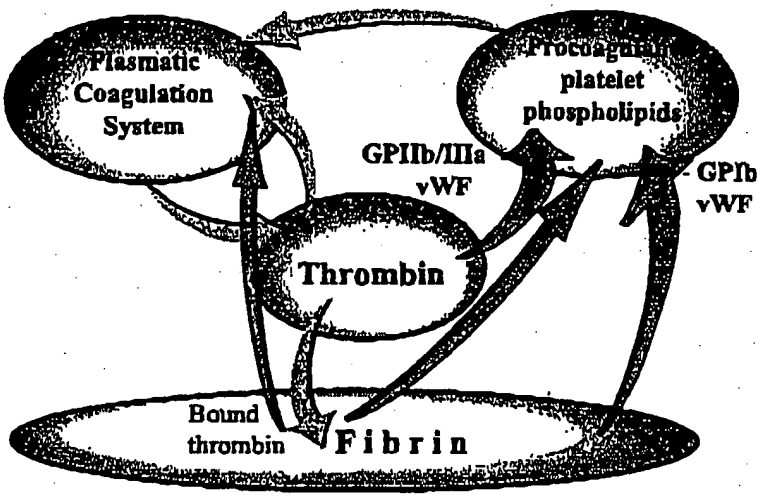
6. Scientific rationale

The HTS is traditionally seen as the result of the sequential action of primary and secondary haemostasis. The first, reflected in the bleeding time, is considered to be a platelet function and its overshoot is the arterial, platelet-rich thrombus. The second is seen as an activity of the plasmatic clotting mechanism; the plasma clotting time is considered the relevant test and venous thrombosis the pathological derailment.

The separation has been artificially stressed because platelet adhesion and aggregation are studied in anticoagulated blood, and clotting in platelet-free plasma. Under these experimental circumstances the co-operation between platelets and coagulation is artificially prevented. The impressive increase of our knowledge of the details of the clotting mechanism and platelet function tends to favour further subdivision. This is reflected in drug research. Not only is a sharp distinction made between anticoagulants and platelet inhibitors, but often a more restricted target is defined (e.g., Factor VIIan or G-proteins) and screening is for an action on that target rather than on global functioning of the HTS.

It is becoming increasingly clear that, *in vivo*, the HTS functions by the concerted action of platelets and plasma, rather than as a consequence of an adhesion-aggregation function of the platelets and a clotting function of the plasma (**Figure 1**). Thrombin is the most potent platelet activator, and platelet activation is essential for thrombin generation.

Figure 1: Outlines of the interactions between the coagulation system and the platelet glycoprotein (GP) IIb/IIIa and GPIb receptors (from[123]).
VWF: von Willebrand factor.



Anticoagulants inhibit arterial as well as venous thrombosis [146,147]. Platelet inhibitors inhibit thrombin generation in PRP, etc.

This is not the place to discuss the mechanisms in detail. We refer to books and review articles for this purpose. Here we only sketch outlines, together with some recent findings that may have an important bearing on drug development.

In the plasmatic coagulation system, Factor Xa is formed by the action of the TF-VIIa. Factor Xa binds to TFPI, and the TFPI-Xa complex inhibits the TF-VIIa complex. Thrombin activates Factors V, VIII and XI and so accelerates its own generation, but it also binds to thrombomodulin and so starts the protein C mechanism that breaks down Factors V and VIII, thus indirectly inhibiting further thrombin generation. An important fraction (~ 30%) of all thrombin formed in clotting plasma is bound to the fibrin clot. Clot-bound thrombin does retain its thrombotic properties; it can clot fibrinogen, activate Factors V, VIII and XI and platelets [123,148,149]. It is not inhibited by AT and, upon fibrinolysis, it may reappear in circulation.

Four different thrombin receptors have been described on the platelet membrane. Thrombin action causes GPIIb/IIIa receptors in the platelet membrane to bind fibrinogen, which causes platelet aggregation. GPIIb/IIIa activation also leads to the exposure of procoagulant phospholipids in a vWF-dependent reaction [123,124]. These phospholipids are required for the proper activation of Factor X and prothrombin. Lately, the picture has been complicated by the discovery that fibrin, previously thought to be the inert end product of coagulation, plays an active role itself. It binds and activates vWF, which in turn activates platelets *via* GPIb, and provokes the exposure of procoagulant phospholipids *via* an alternative pathway.

Thus, the co-operation between platelets and the coagulation system is central to the HTS, and the mechanism shows an abundance of positive and negative (often nested) feedback loops. This necessarily is a non-linear system [150]. In the most simple terms this means that the dose-response relationships become highly unpredictable ('chaotic'). Furthermore, thrombin generation reactions often go to completion, so that classical initial rate kinetics is

of limited applicability. The literature abounds with studies that conjecture on antithrombotic effects of enzyme inhibition on the basis of oversimplified models. Extensive experience in enzyme kinetics tells us that our knowledge of the system (and of non-linear systems in general) is simply insufficient to predict such effects. We may be glad if we can understand certain phenomena *a posteriori*.

An example is the much-debated issue of inhibition of Factor Xa. The original idea was that it was more efficient to inhibit the prothrombin-converting enzyme (Factor Xa) than the product (thrombin) [151,152]. This seemed a very logical idea until it was fully recognised that, under physiological circumstances, Factor Xa requires thrombin-activated Factor V to increase its turnover number [154], and needs adsorption onto procoagulant phospholipid to decrease its K_m below the plasma concentration of prothrombin [153]. The amount of prothrombin-converting complex that can be maximally formed is not higher than the scarcest component. In PRP, with adequate amounts of phospholipid added, it is easily seen that the rate-limiting component is not Factor Xa. The reasons are:

- the plasma concentration of Factor X is approx. 120 nM, whilst that of Factor V is approx. 20 nM, which may rise to 40 nM if the platelets release their Factor V [155]
- the serum concentration of Factor X is considerable ('stable factor'), whereas that of Factor V is near to zero ('labile factor')
- decrease of the Factor Va concentration *via* the APC system efficiently limits prothrombin conversion.

In PRP, the rate limiting component is neither Factor Va nor Factor Xa, but the exposure of procoagulant phospholipids by the platelets [120]. Still closer to real life, if the procoagulant phospholipid surface is much larger than the molecules involved, as in the case of platelets sticking to a wounded surface, the reaction is diffusion-limited and therefore no longer dependent even upon the concentration of the prothrombinase complex on the surface. Only the concentration of prothrombin in the plasma counts [156-158]. Therefore, there are many arguments to show that, in clotting plasma, Factor Xa is available in excess and has to be inhibited by 90% in order to inhibit prothrombinase activity by 50%.

Still, it is often argued that one can expect from direct Factor Xa inhibition an efficacy:safety ratio superior to that achieved by inhibiting thrombin once it is formed. There is no rational theoretical basis for this statement as soon as one realises the importance of thrombin-mediated feedback reactions, and the excess of Factor Xa present in clotting plasma. This does not mean to say that Factor Xa inhibition may not be the mechanism *via* which good antithrombotic efficiency can be obtained. If it is pushed far enough it may certainly cause inhibition of prothrombin conversion. It is only meant as an illustration that predictions on the basis of available theory are precarious. At a previous state of knowledge, one would expect a benefit of Factor Xa inhibition. At present, one would rather predict a narrow therapeutic margin (*viz.* that between 90 - 100% inhibition of Factor X). It is the very essence of this article to show that even sophisticated theoretical arguments cannot predict the *in vivo* behaviour of an antithrombotic, but that a physiological test of the overall function of the HTS is needed instead. Nevertheless, it is a rewarding operation to refute theoretical considerations that are no longer up-to-date, but that tend to survive in pharmaceutical circles.

The proof of the pudding will remain in the eating. It has been shown that thrombin generation can be effectively inhibited by the injection of the pentasaccharide with anti-Factor Xa

activity [159]. This pentasaccharide (Sanofi [SP/90107]; Organon [Org31540]) is now in Phase II clinical trials. DX9065a (Daiichi) is in Phase I clinical trial and TFPI SC59735 is in Phase II. If there are any specific advantages of Factor Xa inhibition they will be seen in such studies.

7. Potential development issues

7.1 Catalysts of physiological inhibition

7.1.1 Purer preparations of the active fractions within natural heparins

Heparins, as they are presently obtained from natural sources, are highly heterogeneous [14]. Apart from the anticoagulant molecules, there is 50 - 80% of other material that is not necessarily without biological action. Also, it has been postulated on good grounds that material with a molecular weight lower than 5400 is much less active than higher molecular weight material [160-162], whereas material larger than about 9000 has an extremely low bioavailability [16]. In addition, it might be more haemorrhagic than lower molecular weight material [18].

7.1.2 Synthetic heparins

It has been an important achievement of Petitou (within the Choay group) to synthesise the pentasaccharide that is responsible for high affinity AT binding in natural heparins. Later research in the Sanofi and Organon teams has led to variants with higher activity [163,164]. These materials have anti-Xa activity only. High doses can be given, however, so that thrombin generation can be inhibited in this way. In view of the much greater efficiency of AT action than of anti-Xa activity, it is to be expected that heparins in which a long inert glycosaminoglycan chain is attached to the active pentasaccharide would make a pure and very active heparin.

7.1.3 Activators of heparin co-Factor II

Dermatan sulfate and pentosan polyphosphate are long since known as antithrombotics [20,164,165]. These drugs have the drawback that concentrations above the micromolar range have to be administered, which at a molecular weight of 10,000 to 100,000 requires administration of large amounts of therapeutic material. The development of pure, low molecular weight material should be considered. There is only 1 μM HCII in plasma. This is lower than the level of prothrombin (2 μM). Optimal activation of all HCII will not exhaust the thrombin-forming capacity of the plasma. Unlike heparin, which acts on 2.4 μM AT, activators of HCII cannot, therefore, be overdosed to cause bleeding.

A special class of substances, the thioxylosides, is capable of altering synthetic reactions in the body of glycosaminoglycans that interact with HCII. Administration of these drugs raises the level of glycosaminoglycans in the plasma, and in this way activate HCII [166,167].

7.2 Direct inhibitors

Enormous progress has been made in our understanding of the stereochemistry of clotting enzymes [168]. This, together with modern techniques of organic synthesis and screening of inhibitory activity [34], has provided us with a number of highly specific inhibitors of individual clotting enzymes, and no doubt more are to come. Sanderson and Naylor-Olsen [169] recently published an excellent review on thrombin inhibitor design, to which we

refer you for details. The hope is that from this line of research a compound will be identified that can be administered orally, and that has pharmacokinetic properties which allow standard dosage without control. Evidently, one of the problems is the large amount of serine proteases present in the intestinal tract.

It will be clear from the above discussion that, in our opinion, there is no predictable relation between the biochemical mode of action of these inhibitors and the pharmacological efficiency. Elg *et al.* compared the kinetic constants of eight different direct thrombin inhibitors and saw that, despite a 50,000-fold difference in K_i value, hirudin and melagatran had similar antithrombotic effects at around 0.14 μM plasma concentration [129]. These authors did report a relation between k_{on} and the slope of the dose-response curve of thrombus inhibition. In theory, a steeper slope indicates a narrower therapeutic interval. To judge inhibitors on this criterion does not seem realistic, however, as long as we are not certain that the pharmacokinetics allow maintenance of stable plasma levels and, more importantly, as long as the dose-response of haemorrhage is not known.

7.3 Platelet inhibitors

At this moment, there are no inhibitors in clinical use that attack *via* GP Ib/IX, the receptor absent in Bernard-Soulier's disease. Antibodies against this inhibitor also strongly (~50%) inhibit thrombin generation. A combination of antibodies against both the GP Ib and the GP IIb/IIIa/IIIa receptors will almost completely abolish thrombin generation in PRP. vWF has been shown to be a necessary co-factor for both receptors; consequently, in the absence of this factor thrombin generation is almost nil, independent of the availability of Factor VIII. This suggests that interfering with these interactions can be an efficient means of antithrombotic action.

We saw that fibrin can play an active role in the development of platelet procoagulant activity, and hence in thrombin generation and further thrombus growth. It may be interesting to try and find inhibitors of the (vWF-mediated) platelet-fibrin interaction, which might lead to early pacification of a growing thrombus.

8. Editorial analysis

In the search for new and better antithrombotics, the open options are as big as the number of vulnerable sites in the HTS (i.e., every enzyme and every receptor involved) multiplied by the number of compounds that can inhibit each site). It is hard to believe that the ideal antithrombotic is not there to be found. The question is how to find it. The present strategy is to first define a subsystem of interest within the HTS; this clotting factor, that membrane receptor. Then a test is set up for that subsystem. Promising compounds are selected on the basis of their performance in that test. Such compounds are then tested in animal thrombosis models and may make it to the phases of investigation in humans.

The problem lies in the choice of the subsystem to be tested. Clotting times and platelet aggregation/adhesion assays are only of limited use because they do not reflect interactions between platelets and the clotting system, recently shown to be extremely important. More specific tests on individual clotting factors or platelet functions are even less fit for the task. The risk is that we find inhibitors of an irrelevant test, and pass by good antithrombotics that do not inhibit this test. The example of LMWHs is a good illustration. These drugs were developed upon the criterion of Factor Xa inhibition, which later was shown to be of no relevance. Later research showed that the performance of these drugs is due to

thrombin-inhibition together with high bioavailability and, probably, absence of large molecular weight molecules that induce bleeding.

Therefore, there is an obvious need for a representative, functional test of the HTS. This test should respond quantitatively in the same manner to any antithrombotic given at a dosage within its therapeutic window. It should, for example, be equally sensitive to doses of antiplatelet drugs and anticoagulants of comparable efficacy.

There are two tests in which the interaction between platelets and plasma is not artificially disrupted: thrombin generation in PRP (or whole blood), and flow chamber experiments in which non-anticoagulated human blood is drawn over a thrombogenic surface. Both tests require much development. In our opinion, progress in technical development of this type of test would be a major step forward in the search for the ideal anticoagulant.

Bibliography

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1. KESSELS H, KESTER AD, HEMKER HC: **Intrinsic and method-induced variation of the bleeding time and related parameters.** *Thromb. Haemost.* (1994) 71:798-799.
2. AGNELLI G: **Thrombin plays a pivotal role in vascular re-occlusion after PTCA and coronary thrombolysis.** *Cardiovasc. Res.* (1996) 31:232-234.
3. BADIMON L, MEYER BJ, BADIMON JJ: **Thrombin in arterial thrombosis.** *Haemostasis* (1994) 24:69-80.
4. CHESEBRO JH, ZOLDHELYI P, BADIMON L, FUSTER V: **Role of thrombin in arterial thrombosis: implications for therapy.** *Thromb. Haemost.* (1991) 66:1-5.
5. CHESEBRO JH, RAUCH U, FUSTER V, BADIMON JJ: **Pathogenesis of thrombosis in coronary artery disease.** *Haemostasis* (1997) 27:12-18.
6. FITZGERALD GA: **The human pharmacology of thrombin inhibition.** [*Coron. Artery Dis.* (1997) 8(1):A4]. *Coron. Artery Dis.* (1996) 7:911-918.
   - • Excellent overview.
7. SCHECTER AD, GIESEN PL, TABY O *et al.*: **Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation.** *J. Clin. Invest.* (1997) 100:2276-2285.
8. TOSCHI V, GALLO R, LETTINO M *et al.*: **Tissue factor modulates the thrombogenicity of human atherosclerotic plaques.** *Circulation* (1997) 95:594-599.
9. WILCOX JN, SMITH KM, SCHWARTZ SM, GORDON D: **Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque.** *Proc. Natl. Acad. Sci. USA* (1989) 86:2839.
10. MARMUR JD, THIRUVIKRAMAN SV, FYFE BS *et al.*: **Identification of active tissue factor in human coronary atheroma.** *Circulation* (1996) 94:1226.
11. HEEMSKERK JW, VUIST WM, FEIJGE MA, REUTELINGSPERGER CP, LINDHOUT T: **Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses.** *Blood* (1997) 90:2615.
12. MURRAY CJ, LOPEZ AD: **Evidence-based health policy - lessons from the Global Burden of Disease Study.** *Science* (1996) 274:740.
13. FUSTER VVM: *Thrombosis in Cardiovascular Disorders.* WB Saunders, Philadelphia, USA (1992).
14. BARROWCLIFFE T: *Low Molecular Weight Heparins.*
    - • Contains essential information on low molecular weight heparins.

15. HEMKER HC, BEGUIN S: The activity of heparin in the presence and absence of Ca^{2+} ions; why the anti-Xa activity of LMW heparins is about two times overestimated. *Thromb. Haemost.* (1993) 70:717.

16. BENDETOWICZ AV, BEGUIN S, CAPLAIN H, HEMKER HC: Pharmacokinetics and pharmacodynamics of a low molecular weight heparin (enoxaparin) after subcutaneous injection, comparison with unfractionated heparin-a three way cross over study in human volunteers. *Thromb. Haemost.* (1994) 71:305.

17. BENDETOWICZ AV, KAI H, KNEBEL R *et al.*: The effect of subcutaneous injection of unfractionated and low molecular weight heparin on thrombin generation in platelet rich plasma - a study in human volunteers. *Thromb. Haemost.* (1994) 72:705.

18. HEMKER HC, BEGUIN S, KAKKAR VV: Can the haemorrhagic component of heparin be identified? Or an attempt at clean thinking on a dirty drug [editorial]. *Haemostasis* (1996) 26:117.

19. TOLLEFSEN DM, BLANK MK: Detection of a new heparin-dependent inhibitor of thrombin in human plasma. *J. Clin. Invest.* (1981) 68:589.

20. DOL F, PETITOU M, CHOAY J, SIE P, HOUIN G, BONEU B: Pharmacological properties of dermatan sulfate, of a low molecular weight dermatan sulfate and of two oversulfated derivatives. *Folia Haematol. Int. Mag. Klin. Morphol. Blutforsch* (1989) 116:851.

21. DOL F, CARANOBE C, DUPOUY D *et al.*: Effects of increased sulfation of dermatan sulfate on its *in vitro* and *in vivo* pharmacological properties. *Thromb. Res.* (1988) 52:153.

22. BENDAYAN P, BOCCALON H, DUPOUY D, BONEU B: Dermatan sulfate is a more potent inhibitor of clot-bound thrombin than unfractionated and low molecular weight heparins. *Thromb. Haemost.* (1994) 71:576.

23. LOSONCZY H, DAVID M, NAGY I: Effect of pentosan polysulfate on activated partial thromboplastin time, thrombin time, euglobulin clot lysis, and on tissue-type plasminogen activator and plasminogen activator inhibitor activities in patients with thromboembolic disease. *Semin. Thromb. Hemost.* (1991) 17:394.

24. MAURAY S, STERNBERG C, THEVENIAUX J *et al.*: Venous antithrombotic and anticoagulant activities of a fucoidan fraction. *Thromb. Haemost.* (1995) 74:1280.

25. SCHENK JF, GLUSA E, RADZIWON P, BUTTI A, MARKWARDT F, BREDDIN HK: A recombinant hirudin (IK-HIR02) in healthy volunteers. II. Effects on platelet adhesion and platelet-induced thrombin generation time. *Haemostasis* (1996) 26:187.

26. SCHENK JF, GLUSA E, RADZIWON P, BUTTI A, MARKWARDT F, BREDDIN HK: A recombinant hirudin (IK-HIR02) in healthy volunteers. I. Effects on coagulation parameters and bleeding time. *Haemostasis* (1996) 26:140.

27. MARKWARDT F: The development of hirudin as an antithrombotic drug. *Thromb. Res.* (1994) 74:1.

28. WALSMANN P: Isolation and characterization of hirudin from *Hirudo medicinalis*. *Semin. Thromb. Hemost.* (1991) 17:83.

29. FITZGERALD D: Specific thrombin inhibitors *in vivo*. *Ann. NY Acad. Sci.* (1994) 714:41.
• Excellent overview.

30. GAST A, TSCHOPP TB, BAUMGARTNER HR: Thrombin plays a key role in late platelet thrombus growth and/or stability. Effect of a specific thrombin inhibitor on thrombogenesis induced by aortic subendothelium exposed to flowing rabbit blood. *Arterioscler. Thromb.* (1994) 14:1466.

31. HERAS M, CHESEBRO JH, PENNY WJ, BAILEY KR, BADIMON L, FUSTER V: Effects of thrombin inhibition on the development of acute platelet- thrombus deposition during angioplasty in pigs. Heparin *versus* recombinant hirudin, a specific thrombin inhibitor. *Circulation* (1989) 79:657.

32. JANG IK, GOLD HK, ZISKIND AA, LEINBACH RC, FALLON JT, COLLEN D: Prevention of platelet-rich arterial thrombosis by selective thrombin inhibition. *Circulation* (1990) 81:219.

33. LEFKOVITS J, TOPOL EJ: Direct thrombin inhibitors in cardiovascular medicine. *Circulation* (1994) 90:1522.

34. LAM KS, SALMON SE, HERSH EM, HRUBY VJ, KAZMIERSKI WM, KNAPP RJ: A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* (1991) 354:82.

35. OSTREM JA, AL-OBEIDI F, SAFAR P *et al.*: Discovery of a novel, potent, and specific family of Factor Xa inhibitors *via* combinatorial chemistry. *Biochemistry* (1998) 37:1053.

36. AROCAS V, ZINGALI RB, GUILLIN MC, BON C, JANDROT-PERRUS M: Bothrojaracin: a potent two-site-directed thrombin inhibitor. *Biochemistry* (1996) 35:9083.

37. CAPPELLO M, BERGUM PW, VLASUK GP, FURMIDGE BA, PRITCHARD DI, AKSOY S: Isolation and characterization of the tsetse thrombin inhibitor: a potent antithrombotic peptide from the saliva of Glossina morsitans morsitans. *Am. J. Trop. Med. Hyg.* (1996) 54:475.

38. FRIEDRICH T, KROGER B, BIALOJAN S *et al.*: A Kazal-type inhibitor with thrombin specificity from *Rhodnius prolixus*. *J. Biol. Chem.* (1993) 268:16216.

39. HOFFMANN A, WALSMANN P, RIESENER G, PAINTZ M, MARKWARDT F: Isolation and characterization of a thrombin inhibitor from the tick ixodes ricinus. *Pharmazie* (1991) 46:209.

40. KODANI S, ISHIDA K, MURAKAMI M: Aeruginosin 103-A, a thrombin inhibitor from the cyanobacterium *Microcystis viridis*. *J. Nat. Prod.* (1998) 61:1046.

41. REBELLO SS, MILLER BV, BASLER GC, LUCCHESI BR: CVS-1123, a direct thrombin inhibitor, prevents occlusive arterial and venous thrombosis in a canine model of vascular injury. *J. Cardiovasc. Pharmacol.* (1997) 29:240.

42. A low molecular weight, selective thrombin inhibitor, inogatran, *vs.* heparin, in unstable coronary artery disease in 1209 patients. A double- blind, randomized, dose-finding study. Thrombin inhibition in Myocardial Ischaemia (TRIM) study group. *Eur. Heart J.* (1997) 18:1416.

43. URIUDA Y, WANG QD, GRIP L, RYDEN L, SJOQUIST PO, MATTSSON C: Antithrombotic activity of inogatran, a new low-molecular-weight inhibitor of thrombin, in a closed-chest porcine model of coronary artery thrombosis. *Cardiovasc. Res.* (1996) 32:320.

44. URIUDA Y, WANG QD, HATORI N *et al.*: Coronary thrombosis/thrombolysis in pigs: effects of heparin, ASA, and the thrombin inhibitor inogatran. *J. Pharmacol. Toxicol. Methods* (1998) 39:81.

45. GUSTAFSSON D, ELG M, LENFORS S, BORJESSON I, TEGER-NILSSON AC: Effects of inogatran, a new low-molecular-weight thrombin inhibitor, in rat models of venous and arterial thrombosis, thrombolysis and bleeding time. *Blood Coagul. Fibrinolysis* (1996) 7:69.

46. GUSTAFSSON D, ANTONSSON T, BYLUND R *et al.*: Effects of melagatran, a new low-molecular-weight thrombin inhibitor, on thrombin and fibrinolytic enzymes. *Thromb. Haemost.* (1998) 79:110.

47. MEHTA JL, CHEN L, NICHOLS WW, MATTSSON C, GUSTAFSSON D, SALDEEN TG: Melagatran, an oral active-site inhibitor of thrombin, prevents or delays formation of electrically induced occlusive thrombus in the canine coronary artery. *J. Cardiovasc. Pharmacol.* (1998) 31:345.

48. ROUX S, TSCHOPP T, BAUMGARTNER HR: Effects of napsagatran (Ro 46-6240), a new synthetic thrombin inhibitor and of heparin in a canine model of coronary artery thrombosis: comparison with an *ex vivo* annular perfusion chamber model. *J. Pharmacol. Exp. Ther.* (1996) 277:71.

• References 13-15: a few examples among many others of thrombin inhibitors.

49. BRADY SF, STAUFFER KJ, LUMMA WC *et al.*: Discovery and development of the novel potent orally active thrombin inhibitor N-(9-hydroxy-9-fluorenecarboxy)prolyl trans-4-aminocyclohexylmethyl amide (L-372,460): coapplication of structure- based design and rapid multiple analogue synthesis on solid support. *J. Med. Chem.* (1998) 41:401.

50. CADROY Y, CARANOBE C, BERNAT A, MAFFRAND JP, SIE P, BONEU B: Antithrombotic and bleeding effects of a new synthetic direct thrombin inhibitor and of standard heparin in the rabbit. *Thromb. Haemost.* (1987) 58:764.

51. CIRILLO R, LIPPI A, SUBISSI A, AGNELLI G, CRISCUOLI M: Experimental pharmacology of hirunorm: a novel synthetic peptide thrombin inhibitor. *Thromb. Haemost.* (1996) 76:384.

52. DESCHENES I, FINKLE CD, WINOCOUR PD: Effective use of BCH-2763, a new potent injectable direct thrombin inhibitor, in combination with tissue plasminogen activator (tPA) in a rat arterial thrombolysis model. *Thromb. Haemost.* (1998) 80:186.

53. FINKLE CD, ST. PIERRE A, LEBLOND L, DESCHENES I, DIMAIO J, WINOCOUR PD: BCH-2763, a novel potent parenteral thrombin inhibitor, is an effective antithrombotic agent in rodent models of arterial and venous thrombosis-comparisons with heparin, r-hirudin, hirulog, inogatran and argatroban. *Thromb. Haemost.* (1998) 79:431.

54. GRIFFIN LC, TOOLE JJ, LEUNG LL: The discovery and characterization of a novel nucleotide-based thrombin inhibitor. *Genc* (1993) 137:25.

55. HARA H, TAMAO Y, KIKUMOTO R, OKAMOTO S: Effect of a synthetic thrombin inhibitor MCI-9038 on experimental models of disseminated intravascular coagulation in rabbits. *Thromb. Haemost.* (1987) 57:165.

56. HIJIKATE A, OKAMOTO S, IKEZAWA K, KIKIMOTO R, TONOMURA S, TAMAO Y: Proceedings: animal experiments of a new synthetic thrombin-inhibitor, dansyl-arginine-methyl-piperidine amide. *Thromb. Diath. Haemorrh.* (1975) 34:347.

57. HUSSAIN MA, KNABB R, AUNGST BJ, KETTNER C: Anticoagulant activity of a peptide boronic acid thrombin inhibitor by various routes of administration in rats. *Peptides* (1991) 12:1153.

58. IKOMA H, OHTSU K, TAMAO Y *et al.*: Effect of a potent thrombin inhibitor, No. 407, on novel experimental thrombosis generated by acetic acid. *Kobe J. Med. Sci.* (1980) 26:33.

59. KAISER B, HAUPTMANN J, WEISS A, MARKWARDT F: Pharmacological characterization of a new highly effective synthetic thrombin inhibitor. *Biomed. Biochim. Acta* (1985) 44:1201.

60. OKAYAMA T, ARAKI S, MIYAMAE T, MORITA Y, MORIKAWA T, HAGIWARA M: Anticoagulant activity of the novel thrombin inhibitor 1-butyl-3-(6,7-dimethoxy-2-naphthylsulfonyl) amino-3-(3-guanidinopropyl)-2- pyrrolidinone hydrochloride. *Arzneimittelforschung* (1997) 47:1023.

61. NOESKE-JUNGBLUT C, HAENDLER B, DONNER P, ALAGON A, POSSANI L, SCHLEUNING WD: Triabin, a highly potent exosite inhibitor of thrombin. *J. Biol. Chem.* (1995) 270:28629.

62. RUPIN A, MENNECIER P, LILA C, DE NANTEUIL G, VERBEUREN TJ: Selection of S18326 as a new potent and selective boronic acid direct thrombin inhibitor. *Thromb. Haemost.* (1997) 78:1221.

63. SHUMAN RT, GESELLCHEN PD: Development of an orally active tripeptide arginal thrombin inhibitor. *Pharm. Biotechnol.* (1998) 11:57.

64. TAPPARELLI C, METTERNICH R, EHRHARDT C *et al.*: *In vitro* and *in vivo* characterization of a neutral boron-containing thrombin inhibitor. *J. Biol. Chem.* (1993) 268:4734.

65. TREMOLI E, COLLI S, PAOLETTI R: GYKI 14,451, a synthetic tripeptide inhibitor of thrombin: *'in vitro'* and *'in vivo'* studies. *Adv. Exp. Med. Biol.* (1984) 164:187.

66. BAGDY D, BARABAS E, FITTLER Z *et al.*: Experimental oral anticoagulation by a directly acting thrombin inhibitor (RGH-2958). *Folia Haematol. Int. Mag. Klin. Morphol. Blutforsch* (1988) 115:136.

67. ANDERSEN K, DELLBORG M: Heparin is more effective than inogatran, a low-molecular weight thrombin inhibitor in suppressing ischemia and recurrent angina in unstable coronary disease. Thrombin Inhibition in Myocardial Ischemia (TRIM) Study Group. *Am. J. Cardiol.* (1998) 81:939.

68. LUNVEN C, GAUFFENY C, LECOFFRE C, O'BRIEN DP, ROOME NO, BERRY CN: Inhibition by Argatroban, a specific thrombin inhibitor, of platelet activation by fibrin clot-associated thrombin. *Thromb. Haemost.* (1996) 75:154.

69. APITZ-CASTRO R, BEGUIN S, TABLANTE A, BARTOLI F, HOLT JC, HEMKER HC: **Purification and partial characterization of draculin, the anticoagulant factor present in the saliva of vampire bats (*Desmodus rotundus*).** *Thromb. Haemost.* (1995) 73:94.

70. DUNWIDDIE C, THORNBERRY NA, BULL HG *et al.*: **Antistasin, a leech-derived inhibitor of Factor Xa. Kinetic analysis of enzyme inhibition and identification of the reactive site.** *J. Biol. Chem.* (1989) 264:16694.

• References 20 & 21; two among the many natural Factor Xa inhibitors.

71. DUNWIDDIE CT, SMITH DE, NUTT EM, VLASUK GP: **Anticoagulant effects of the selective Factor XA inhibitors tick anticoagulant peptide and antistasin in the APTT assay are determined by the relative rate of prothrombinase inhibition.** *Thromb. Res.* (1991) 64:787.

72. DUNWIDDIE CT, NEEPER MP, NUTT EM *et al.*: **Site-directed analysis of the functional domains in the Factor Xa inhibitor tick anticoagulant peptide: identification of two distinct regions that constitute the enzyme recognition sites.** *Biochemistry* (1992) 31:12126.

73. HAUPTMANN J, KAISER B: **Anticoagulant and antithrombotic action of the Factor Xa inhibitor antistasin (ATS).** *Thromb. Res.* (1993) 71:169.

74. HERAULT JP, BERNAT A, PFLIEGER AM, LORMEAU JC, HERBERT JM: **Comparative effects of two direct and indirect Factor Xa inhibitors on free and clot-bound prothrombinase.** *J. Pharmacol. Exp. Ther.* (1997) 283:16.

75. JORDAN SP, WAXMAN L, SMITH DE, VLASUK GP: **Tick anticoagulant peptide: kinetic analysis of the recombinant inhibitor with blood coagulation Factor Xa.** *Biochemistry* (1990) 29:11095.

76. JORDAN SP, MAO SS, LEWIS SD, SHAFER JA: **Reaction pathway for inhibition of blood coagulation Factor Xa by tick anticoagulant peptide.** *Biochemistry* (1992) 31:5374.

77. KATAKURA S, NAGAHARA T, HARA T, IWAMOTO M: **A novel Factor Xa inhibitor: structure-activity relationships and selectivity between Factor Xa and thrombin.** *Biochem. Biophys. Res. Commun.* (1993) 197:965.

78. MAO SS, HUANG J, WELEBOB C, NEEPER MP, GARSKY VM, SHAFER JA: **Identification and characterization of variants of tick anticoagulant peptide with increased inhibitory potency toward human Factor Xa.** *Biochemistry* (1995) 34:5098.

79. MORISHIMA Y, TANABE K, TERADA Y, HARA T, KUNITADA S: **Antithrombotic and hemorrhagic effects of DX-9065a, a direct and selective Factor Xa inhibitor: comparison with a direct thrombin inhibitor and antithrombin III-dependent anticoagulants.** *Thromb. Haemost.* (1997) 78:1366.

80. NUTT E, GASIC T, RODKEY J *et al.*: **The amino acid sequence of antistasin. A potent inhibitor of Factor Xa reveals a repeated internal structure.** *J. Biol. Chem.* (1988) 263:10162.

81. NUTT EM, JAIN D, LENNY AB, SCHAFFER L, SIEGL PK, DUNWIDDIE CT: **Purification and characterization of recombinant antistasin: a leech- derived inhibitor of coagulation Factor Xa.** *Arch. Biochem. Biophys.* (1991) 285:37.

82. PRASA D, SVENDSEN L, STURZEBECHER J: **Inhibition of thrombin generation in plasma by inhibitors of Factor Xa.** *Thromb. Haemost.* (1997) 78:1215.

83. SCHREUDER H, ARKEMA A, DE BOER B *et al.*: **Crystallization and preliminary crystallographic analysis of antistasin, a leech-derived inhibitor of blood coagulation Factor Xa.** *J. Mol. Biol.* (1993) 231:1137.

84. VLASUK GP: **Structural and functional characterization of tick anticoagulant peptide (TAP): a potent and selective inhibitor of blood coagulation Factor Xa.** *Thromb. Haemost.* (1993) 70:212.

85. WAXMAN L, SMITH DE, ARCURI KE, VLASUK GP: **Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation Factor Xa.** *Science* (1990) 248:593.

86. YAMAZAKI M, ASAKURA H, AOSHIMA K *et al.*: **Effects of DX-9065a, an orally active, newly synthesized and specific inhibitor of Factor Xa, against experimental disseminated intravascular coagulation in rats.** *Thromb. Haemost.* (1994) 72:392.

87. JOHNSON K, HUNG D: **Novel anticoagulants based on inhibition of the Factor VIIa/tissue factor pathway.** *Coron. Artery Dis.* (1998) 9:83.

88. HUANG M, SYED R, STURA EA *et al.*: The mechanism of an inhibitory antibody on TF-initiated blood coagulation revealed by the crystal structures of human tissue factor, Fab 5G9 and TF.G9 complex. *J. Mol. Biol.* (1998) 275:873.

89. HARKER LA, HANSON SR, WILCOX JN, KELLY AB: Antithrombotic and antilesion benefits without hemorrhagic risks by inhibiting tissue factor pathway. *Haemostasis* (1996) 26:76.

90. ORVIM U, BARSTAD RM, ORNING L *et al.*: Antithrombotic efficacy of inactivated active site recombinant Factor VIIa is shear dependent in human blood. *Arterioscler. Thromb. Vasc. Biol.* (1997) 17:3049.

91. KJALKE M, OLIVER JA, MONROE DM *et al.*: The effect of active site-inhibited Factor VIIa on tissue factor- initiated coagulation using platelets before and after aspirin administration. *Thromb. Haemost.* (1997) 78:1202.

92. RAGNI M, GOLINO P, CIRILLO P *et al.*: Inactivated Factor VII exercises a powerful antithrombotic activity in an experimental model of recurrent arterial thrombosis. *Cardiologia* (1996) 41:51.

93. RONNING HF, RISOEN UC, ORNING L, SLETTEN K, SAKARIASSEN KS: Synthetic peptide analogs of tissue factor and Factor VII which inhibit Factor Xa formation by the tissue factor/Factor VIIa complex. *Thromb. Res.* (1996) 84:73.

94. RAGOSTA M, GIMPLE LW, GERTZ SD *et al.*: Specific Factor Xa inhibition reduces restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. *Circulation* (1994) 89:1262.

95. LEFKOVITS J, MALYCKY JL, RAO JS *et al.*: Selective inhibition of Factor Xa is more efficient than Factor VIIa- tissue factor complex blockade at facilitating coronary thrombolysis in the canine model. *J. Am. Coll. Cardiol.* (1996) 28:1858.

96. BENEDICT CR, RYAN J, TODD J *et al.*: Active site-blocked Factor Xa prevents thrombus formation in the coronary vasculature in parallel with inhibition of extravascular coagulation in a canine thrombosis model. *Blood* (1993) 81:2059.

97. FITZGERALD GA, MEAGHER EA: Antiplatelet drugs. *Eur. J. Clin. Invest.* (1994) 24(Suppl. 1):46.

98. VERSTRAETE M: Modulating platelet function with selective thrombin inhibitors. *Haemostasis* (1996) 26(Suppl 4):70.

99. MONCADA S, VANE JR: Arachidonic acid metabolites and the interactions between platelets and blood-vessel walls. *New Engl. J. Med.* (1979) 300:1142.

100. FITZGERALD GA: Dipyridamole. *New Engl. J. Med.* (1987) 316:1247.

101. GRESELE P, ARNOUT J, DECKMYN H, VERMYLEN J: Mechanism of the antiplatelet action of dipyridamole in whole blood: modulation of adenosine concentration and activity. *Thromb. Haemost.* (1986) 55:12.

102. OATES JA, FITZGERALD GA, BRANCH RA, JACKSON EK, KNAPP HR, ROBERTS LJD: Clinical implications of prostaglandin and thromboxane A2 formation (1). *New Engl. J. Med.* (1988) 319:689.

103. OATES JA, FITZGERALD GA, BRANCH RA, JACKSON EK, KNAPP HR, ROBERTS LJD: Clinical implications of prostaglandin and thromboxane A2 formation (2). *New Engl. J. Med.* (1988) 319:761.

104. NICOLINI FA, MEHTA JL, NICHOLS WW, SALDEEN TG, GRANT M: Prostacyclin analogue iloprost decreases thrombolytic potential of tissue-type plasminogen activator in canine coronary thrombosis. *Circulation* (1990) 81:1115.

105. TOPOL EJ, ELLIS SG, CALIFF RM *et al.*: Combined tissue-type plasminogen activator and prostacyclin therapy for acute myocardial infarction. Thrombolysis and Angioplasty in Myocardial Infarction (TAMI) 4 Study Group. *J. Am. Coll. Cardiol.* (1989) 14:877.

106. DARIUS H, NIXDORFF U, ZANDER J, RUPPRECHT HJ, ERBEL R, MEYER J: Effects of ciprostene on restenosis rate during therapeutic transluminal coronary angioplasty. *Agents Actions Suppl.* (1992) 37:305.

107. FIDDLER GI, LUMLEY P: Preliminary clinical studies with thromboxane synthase inhibitors and thromboxane receptor blockers. A review. *Circulation* (1990) 81:I69.

108. VERSTRAETE M: The PACK trial: morbidity and mortality effects of ketanserin. Prevention of Atherosclerotic Complications. *Vasc. Med.* (1996) 1:135.

109. Prevention of atherosclerotic complications: controlled trial of ketanserin. Prevention of Atherosclerotic Complications with Ketanserin Trial Group. *Br. Med. J.* (1989) 298:424.

110. DEFREYN G, BERNAT A, DELEBASSEE D, MAFFRAND JP: Pharmacology of ticlopidine: a review. *Semin. Thromb. Hemost.* (1989) 15:159.

111. BALSANO F, RIZZON P, VIOLI F *et al.*: Antiplatelet treatment with ticlopidine in unstable angina. A controlled multicenter clinical trial. The Studio della Ticlopidina nell'Angina Instabile Group. *Circulation* (1990) 82:17.

112. BOSSAVY JP, THALAMAS C, SAGNARD L *et al.*: A double-blind randomized comparison of combined aspirin and ticlopidine therapy *versus* aspirin or ticlopidine alone on experimental arterial thrombogenesis in humans. *Blood* (1998) 92:1518.

113. HERBERT JM, BERNAT A, SAMAMA M, MAFFRAND JP: The anti-aggregating and antithrombotic activity of ticlopidine is potentiated by aspirin in the rat. *Thromb. Haemost.* (1996) 76:94.

114. ROALD HE, BARSTAD RM, KIERULF P *et al.*: Clopidogrel-a platelet inhibitor which inhibits thrombogenesis in non-anticoagulated human blood independently of the blood flow conditions. *Thromb. Haemost.* (1994) 71:655.

115. TAYLOR FB, COLLER BS, CHANG AC *et al.*: 7E3 F(ab')2, a monoclonal antibody to the platelet GPIIb/IIIa receptor, protects against microangiopathic hemolytic anemia and microvascular thrombotic renal failure in baboons treated with C4b binding protein and a sublethal infusion of *Escherichia coli*. *Blood* (1997) 89:4078.

116. AMMAR T, SCUDDER LE, COLLER BS: *In vitro* effects of the platelet glycoprotein IIb/IIIa receptor antagonist c7E3 Fab on the activated clotting time. *Circulation* (1997) 95:614.

117. COLLER BS, ANDERSON KM, WEISMAN HF: The anti-GPIIb-IIIa agents: fundamental and clinical aspects. *Haemostasis* (1996) 26(Suppl. 4):285.

118. GOLD HK, COLLER BS, YASUDA T *et al.*: Rapid and sustained coronary artery recanalization with combined bolus injection of recombinant tissue-type plasminogen activator and monoclonal antiplatelet GPIIb/IIIa antibody in a canine preparation. *Circulation* (1988) 77:670.

119. COLLER BS: A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. *J. Clin. Invest.* (1985) 76:101.

120. BEGUIN S, LINDHOUT T, HEMKER HC: The effect of trace amounts of tissue factor on thrombin generation in platelet rich plasma, its inhibition by heparin. *Thromb. Haemost.* (1989) 61:25.

121. KEULARTS IM, BEGUIN S, DE ZWAAN C, HEMKER HC: Treatment with a GPIIb/IIIa antagonist inhibits thrombin generation in platelet rich plasma from patients. *Thromb. Haemost.* (1998) 80:370.

122. REVERTER JC, BEGUIN S, KESSELS H, KUMAR R, HEMKER HC, COLLER BS: Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and 'clinical restenosis'. *J. Clin. Invest.* (1996) 98:863.
• First demonstration that an anti-aggregant does influence thrombin generation.

123. BEGUIN S, KUMAR R: Thrombin, fibrin and platelets: a resonance loop in which von Willebrand factor is a necessary link. *Thromb. Haemost.* (1997) 78:590.

124. BÉGUIN S, KUMAR R, KEULARTS I, SELIGSOHN U, COLLER BS, HEMKER HC: Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. (1999) *Blood* 93:564-70.
• Demonstration that fibrin is thrombogenic and acts through von Willebrand factor.

125. CHIU HM, HIRSH J, YUNG WL, REGOECZI E, GENT M: Relationship between the anticoagulant and antithrombotic effects of heparin in experimental venous thrombosis. *Blood* (1977) 49:171.

126. BASU D, GALLUS A, HIRSH J, CADE J: A prospective study of the value of monitoring heparin treatment with the activated partial thromboplastin time. *New Engl. J. Med.* (1972) 287:324.

127. HULL RD, RASKOB GE, HIRSH J *et al.*: Continuous intravenous heparin compared with intermittent subcutaneous heparin in the initial treatment of proximal-vein thrombosis. *New Engl. J. Med.* (1986) 315:1109.

128. ANAND S, GINSBERG JS, KEARON C, GENT M, HIRSH J: The relation between the activated partial thromboplastin time response and recurrence in patients with venous thrombosis treated with continuous intravenous heparin. *Arch. Intern. Med.* (1996) 156:1677.

129. ELG M, GUSTAFSSON D, DEINUM J: The importance of enzyme inhibition kinetics for the effect of thrombin inhibitors in a rat model of arterial thrombosis. *Thromb. Haemost.* (1997) 78:1286.

130. POLLER L, VAN DEN BESSELAAR AM, JESPERSEN J, TRIPODI A, HOUGHTON D: The European Concerted Action on Anticoagulation (ECAA): field studies of coagulometer effects on the ISI of ECAA thromboplastins. *Thromb. Haemost.* (1998) 80:615.

131. NOWAK G, BUCHA E: Quantitative determination of hirudin in blood and body fluids. *Semin. Thromb. Hemost.* (1996) 22:197.

132. BERRY CN, LUNVEN C, GIRARDOT C *et al.*: Ecarin clotting time: a predictive coagulation assay for the antithrombotic activity of argatroban in the rat. *Thromb. Haemost.* (1998) 79:228.

133. SAKARIASSEN KS, AARTS PA, DE GROOT PG, HOUDIJK WP, SIXMA JJ: A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. *J. Lab. Clin. Med.* (1983) 102:522.

134. SAKARIASSEN KS, KUHN H, MUGGLI R, BAUMGARTNER HR: Growth and stability of thrombi in flowing citrated blood: assessment of platelet-surface interactions with computer-assisted morphometry. *Thromb. Haemost.* (1988) 60:392.

135. HANSON SR, SAKARIASSEN KS: Blood flow and antithrombotic drug effects. *Am. Heart J.* (1998) 135:S132.

136. BADIMON L, BADIMON JJ, GALVEZ A, CHESEBRO JH, FUSTER V: Influence of arterial damage and wall shear rate on platelet deposition. *Ex vivo* study in a swine model. *Arteriosclerosis* (1986) 6:312.

137. BOSSAVY JP, SAKARIASSEN KS, BARRET A, BONEU B, CADROY Y: A new method for quantifying platelet deposition in flowing native blood in an *ex vivo* model of human thrombogenesis. *Thromb. Haemost.* (1998) 79:162.

138. BARSTAD RM, ROALD HE, CUI Y, TURITTO VT, SAKARIASSEN KS: A perfusion chamber developed to investigate thrombus formation and shear profiles in flowing native human blood at the apex of well- defined stenoses. *Arterioscler. Thromb.* (1994) 14:1984.

139. HOLME PA, ORVIM U, HAMERS MJ *et al.*: Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis. *Arterioscler. Thromb. Vasc. Biol.* (1997) 17:646.

140. DIQUELOU A, LEMOZY S, DUPOUY D, BONEU B, SAKARIASSEN K, CADROY Y: Effect of blood flow on thrombin generation is dependent on the nature of the thrombogenic surface. *Blood* (1994) 84:2206.

141. HEMKER HC, WILLEMS GM, BEGUIN S: A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb. Haemost.* (1986) 56:9.

142. HEMKER HC, BEGUIN S: Thrombin generation in plasma: its assessment *via* the endogenous thrombin potential. *Thromb. Haemost.* (1995) 74:134.

143. HERAULT JP, PEYROU V, SAVI P, BERNAT A, HERBERT JM: **Effect of SR121566A, a potent GP IIb-IIIa antagonist on platelet- mediated thrombin generation *in vitro* and *in vivo*.** *Thromb. Haemost.* (1998) 79:383.

144. PEYROU V, BEGUIN S, BONEU B, HEMKER HC: **The activity of unfractionated heparin and low molecular weight heparins in rabbit plasma-the need for using absolute anti-Factor Xa and antithrombin activities.** *Thromb. Haemost.* (1997) 77:312.

145. WIELDERS S, MUKHERJEE M, MICHIELS J *et al.*: **The routine determination of the endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability.** *Thromb. Haemost.* (1997) 77:629.

146. **A double-blind trial to assess long-term oral anticoagulant therapy in elderly patients after myocardial infarction. Report of the Sixty Plus Reinfarction Study Research Group.** *Lancet* (1980) 2:989.

147. NERI SERNERI GG, ROVELLI F, GENSINI GF, PIRELLI S, CARNOVALI M, FORTINI A: **Effectiveness of low-dose heparin in prevention of myocardial reinfarction.** *Lancet* (1987) 1:937.

• References 46 and 47 together demonstrate that thrombin inhibition inhibits arterial thrombi, independent of the mechanism of thrombin inhibition..

148. KUMAR R, BEGUIN S, HEMKER HC: **The influence of fibrinogen and fibrin on thrombin generation-evidence for feedback activation of the clotting system by clot bound thrombin.** *Thromb. Haemost.* (1994) 72:713.

149. KUMAR R, BEGUIN S, HEMKER HC: **The effect of fibrin clots and clot-bound thrombin on the development of platelet procoagulant activity.** *Thromb. Haemost.* (1995) 74:962.

150. HEMKER HC, HESS B: *Analysis and Simulation of Biochemical Systems.* Elsevier, Amsterdam, Holland (1972).

151. YIN ET, WESSLER S, STOLL PJ: **Biological properties of the naturally occurring plasma inhibitor to activated Factor X.** *J. Biol. Chem.* (1971) 246:3703.

152. WESSLER S, YIN ET: **Theory and practice of minidose heparin in surgical patients. A status report.** *Circulation* (1973) 47:671.

153. ROSING J, TANS G, GOVERS-RIEMSLAG JW, ZWAAL RF, HEMKER HC: **The role of phospholipids and Factor Va in the prothrombinase complex.** *J. Biol. Chem.* (1980) 255:274.

154. PIETERS J, LINDHOUT T, HEMKER HC: ***In situ*-generated thrombin is the only enzyme that effectively activates Factor VIII and Factor V in thromboplastin-activated plasma.** *Blood* (1989) 74:1021.

155. BARUCH D, HEMKER HC, LINDHOUT T: **Kinetics of thrombin-induced release and activation of platelet Factor V.** *Eur. J. Biochem.* (1986) 154:213.

156. BILLY D, SPEIJER H, LINDHOUT T, HEMKER HC, WILLEMS GM: **Inhibition of prothrombinase at macroscopic lipid membranes: competition between antithrombin and prothrombin.** *Biochemistry* (1995) 34:13699.

157. BILLY D, SPEIJER H, WILLEMS G, HEMKER HC, LINDHOUT T: **Prothrombin activation by prothrombinase in a tubular flow reactor.** *J. Biol. Chem.* (1995) 270:1029.

158. BILLY D, BRIEDE J, HEEMSKERK JW, HEMKER HC, LINDHOUT T: **Prothrombin conversion under flow conditions by prothrombinase assembled on adherent platelets.** *Blood Coagul. Fibrinolysis* (1997) 8:168.

159. LORMEAU JC, HERAULT JP: **The effect of the synthetic pentasaccharide SR 90107/ORG 31540 on thrombin generation *ex vivo* is uniquely due to ATIII-mediated neutralization of Factor Xa.** *Thromb. Haemost.* (1995) 74:1474.

160. BEGUIN S, LINDHOUT T, HEMKER HC: **The mode of action of heparin in plasma.** *Thromb. Haemost.* (1988) 60:457.

• Demonstration that anti-Factor Xa activity of heparin contributes little to heparin action.

161. BEGUIN S, MARDIGUIAN J, LINDHOUT T, HEMKER HC: **The mode of action of low molecular weight heparin preparation (PK10169) and two of its major components on thrombin generation in plasma.** *Thromb. Haemost.* (1989) 61:30.

162. BEGUIN S, WIELDERS S, LORMEAU JC, HEMKER HC: **The mode of action of CY216 and CY222 in plasma.** *Thromb. Haemost.* (1992) 67:33.

163. PETITOU M, DUCHAUSSOY P, JAURAND G *et al.*: **Synthesis and pharmacological properties of a close analogue of an antithrombotic pentasaccharide (SR 90107A/ORG 31540).** *J. Med. Chem.* (1997) 40:1600.

164. PETITOU M, DUCHAUSSOY P, LEDERMAN I *et al.*: **Synthesis of heparin fragments. A chemical synthesis of the pentasaccharide O-(2-deoxy-2-sulfamido-6-O-sulfo-alpha-D-glucopyranosyl)-(1-4)-O-(beta-D-glucopyranosyluronic acid)-(1-4)-O-(2-deoxy-2-sulfamido-3,6-di-O-sulfo-alpha-D-glu copyranosyl)-(1-4)-O-(2-O-sulfo-alpha-L-idopyranosyluronic acid)-(1-4)-2-deoxy-2-sulfamido-6-O-sulfo-D-glucopyranose decasodium salt, a heparin fragment having high affinity for antithrombin III.** *Carbohydr. Res.* (1986) 147:221.
- The first synthesis of an active polysaccharide with heparin-action.

165. DOL F, PETITOU M, LORMEAU JC *et al.*: **Pharmacologic properties of a low molecular weight dermatan sulfate: comparison with unfractionated dermatan sulfate.** *J. Lab. Clin. Med.* (1990) 115:43.

166. AGUEJOUF O, OUALANE FA, INAMO J *et al.*: **The arterial antithrombotic activity of thioxylosides in a rat model of laser-induced thrombosis.** *Semin. Thromb. Hemost.* (1996) 22:327.

167. BELLAMY F, HORTON D, MILLET J, PICART F, SAMRETH S, CHAZAN JB: **Glycosylated derivatives of benzophenone, benzhydrol, and benzhydril as potential venous antithrombotic agents.** *J. Med. Chem.* (1993) 36:898.

168. BODE W, MAYR I, BAUMANN U, HUBER R, STONE SR, HOFSTEENGE J: **The refined 1.9 A crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment.** *Embo. J.* (1989) 8:3467.
- Classical paper on which much of the stereochemistry of thrombin inhibitors is based.

169. SANDERSON PE, NAYLOR-OLSEN AM: **Thrombin inhibitor design.** *Curr. Med. Chem.* (1998) 5:289.

170. GIESSEN *PLA et al.*: **Blood borne tissue factor: a new view on thrombosis.** *Proc. Natl. Acad. Sci. USA* (In Press).

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ratory animals. Bethesda, Md.: National Institutes of Health; 1985; NIH publication no 85-23.
18. Buckberg GD, Luck JC, Payne BE, Hoffman JIE, Archie JP, Fixler DE. Some sources of error in measuring regional blood flow with radioactive microspheres. J Appl Physiol 1971;31:598-604.
19. Cohen RA, Shepherd JT, Vanhoutte PM. Inhibitory role of the endothelium in the response of isolated coronary arteries to platelets. Science 1983;221:273-4.
20. Benedict CR, Mathew B, Rex KA, Cartwright J Jr, Sordahl LA. Correlation of plasma serotonin changes with platelet aggregation in an *in vivo* dog model of spontaneous occlusive coronary thrombus formation. Circ Res 1986;58:58-67.
21. Mena MA, Vidrio H. On the mechanism of the coronary dilator effect of serotonin in the dog. Eur J Pharm 1976;36:1-5.
22. Houston DS, Shepherd JT, Vanhoutte PM. Adenine nucleotides, serotonin, and endothelium dependent relaxations to platelets. Am J Physiol 1985;248:H389-95.
23. Vanhoutte PM, Shimokawa H. Endothelium-derived relaxing factor and coronary vasospasm. Circulation 1989;80:1-9.
24. Mankad PS, Chester AH, Yacoub MH. 5-Hydroxytryptamine mediates endothelium dependent coronary vasodilation in the isolated rat heart by the release of nitric oxide. Cardiovasc Res 1991;25:24-8.

Neutrophil activation after percutaneous transluminal coronary angioplasty

We investigated whether percutaneous transluminal coronary angioplasty (PTCA) would induce neutrophil activation in patients with coronary artery disease. Blood samples were taken from the coronary sinus in 14 patients who underwent PTCA and in 9 control subjects who underwent coronary arteriography (CAG). Flow cytometry was used to measure membrane surface expression of β_2 integrin (CD11b) and the generation of hydrogen peroxide in neutrophils after ex vivo phorbol myristate acetate stimulation by 2,'7'-dichlorofluorescein. Neutrophil elastase was measured by an immunoenzymatic method. Surface expression of CD11b increased significantly, approximately twofold, after PTCA but not after CAG. Mean fluorescence intensity of 2',7'-dichlorofluorescein in stimulated neutrophils decreased significantly after PTCA, suggesting a previous in vivo activation, but not after CAG. Neutrophil elastase increased significantly after PTCA but not after CAG. These data indicate that PTCA induces neutrophil activation and suggest that neutrophils may contribute to the ischemic injury. (Am Heart J 1994;128:1091-8.)

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Neutrophil activation appears to be a component of the atherosclerotic process involving the vascular wall[1, 2] and of ischemia–reperfusion myocardial injury.[3, 4] Activated neutrophils can release a variety of mediators that are potentially deleterious to the heart, such as oxygen-derived free radicals, proteolytic enzymes, and oxygenase-derived autacoids that are capable of inducing platelet aggregation, vasoconstriction, and direct inflammatory tissue damage. Indeed studies have demonstrated that ischemic myocardial injury is reduced by experimentally depletion of neutrophils by antineutrophil antibodies or filters or by antiinflammatory agents that alter neutrophil function.[5, 6] Neutrophils may thus exert direct cytotoxic effects on coronary vascular and myocardial cells.

Percutaneous transluminal coronary angioplasty (PTCA) is widely accepted for treating patients with

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Table I. Clinical profile of patients undergoing percutaneous transluminal coronary angioplasty

Patient	Sex	Age (yr)	Dilated vessel	Stenosis (%)		Inflation time (sec)	ST-Segment changes during inflation
				Before PTCA	After PTCA		
1	M	60	LAD	77	36	120	No change
2	M	58	LAD	84	39	120	Elevation
3	M	54	Cx	100	31	240	No change
4	M	59	LAD	87	36	150	Elevation
5	M	52	LAD	94	47	180	No change
6	M	58	LAD	82	34	270	Elevation
7	M	68	Cx	80	36	210	Depression
8	M	62	LAD	86	42	270	Elevation
9	M	61	LAD	85	23	150	Elevation
10	M	41	LAD	100	46	360	No change
11	M	67	LAD	90	40	90	Elevation
12	M	55	LAD	86	38	120	Depression
13	M	52	LAD	81	47	180	Depression
14	F	62	Cx	79	40	180	Depression
Mean ± SE		57.8 ± 1.8		86.5 ± 1.9	38.2 ± 1.7*	188.6 ± 20.0	

Cx, Circumflex coronary artery; *LAD*, left anterior descending coronary artery; *PTCA*, percutaneous transluminal coronary angioplasty.
*$p < 0.0001$ compared with before PTCA.

coronary artery disease. PTCA can be regarded as a clinical model of ischemia–reperfusion because its procedures are characterized by successive short periods of myocardial ischemia. The purpose of this study was to examine whether PTCA induces neutrophil activation associated with the membrane surface expression of β_2 integrin, the generation of hydrogen peroxide, and the release of proteolytic enzymes by activated neutrophils.

METHODS

Patients. The study group consisted of 14 patients with one-vessel coronary artery disease scheduled for an elective PTCA (Table I). These 13 men and 1 woman ranged in age from 41 to 68 (mean 58) years. Ten patients had a history of effort angina pectoris with no evidence of a previous infarction, and 4 patients had had a non–Q-wave infarction (>6 weeks before the study). All patients had a viable myocardium as defined by the development of typical chest pain with ST-segment depression of ≥1 mm during an exercise stress test or a reversible perfusion defect on exercise thalium-201 scintigraphy. Of 9 patients who received routine coronary arteriography, 4 had angina pectoris and 5 had had Q-wave infarction (>6 weeks before the study). These patients served as a control group and included 7 men and 2 women whose age ranged from 45 to 69 (mean 59) years. Written informed consent was obtained from all subjects.

Protocol. All medications, including aspirin, nitrate, calcium-channel blocking agents, and β-adrenergic blocking agents were discontinued on the day of the procedure. After premedication with diazepam and local anesthesia, a 7F catheter was advanced from an antecubital vein to the coronary sinus. A sheath was placed into a femoral artery for insertion of coronary angiographic and angioplastic catheters. Before the intracoronary manipulations were begun, an intravenous bolus of 5000 IU heparin was administered. All patients received isosorbide dinitrate during the procedure. Heparinized blood samples were obtained at the beginning of the procedure from the coronary sinus and from the guiding catheter in the aorta. Coronary angioplasty was performed with balloon dilation catheters of 2.0 to 3.5 mm diameter; balloon size was chosen for each patient based on the diameter of normal segments adjacent to the stenosis. The balloon was inflated for 45 to 90 seconds at a pressure of 4 to 8 atm. PTCA was regarded as successful when the stenotic segment was dilated so that luminal narrowing was <50%. No significant complications related to the procedure occurred.

Blood samples were obtained from the coronary sinus immediately after the PTCA procedure. Coronary artery diameter was measured with calipers at the site of maximal obstruction and at the proximal and distal portions of the obstruction, and percent luminal narrowing was calculated.

The nine control patients underwent routine coronary arteriography. All cardiovascular medications were discontinued on the day of the procedure. These patients received an intravenous injection of 4000 IU heparin and isosorbide dinitrate during the procedure. Coronary arteriography was performed by Sones' technique. A 7F catheter was advanced from an antecubital vein to the coronary sinus. Heparinized blood samples were obtained from the coronary sinus and from the aorta at the beginning and the end of the procedure. Blood was sampled at times corresponding to those used in the PTCA procedure.

Flow-cytometric single-cell analysis. Neutrophil surface expression of CD11b was measured by flow cytometry. Heparinized blood was obtained from the coronary sinus immediately before and after the PTCA or coronary arte-

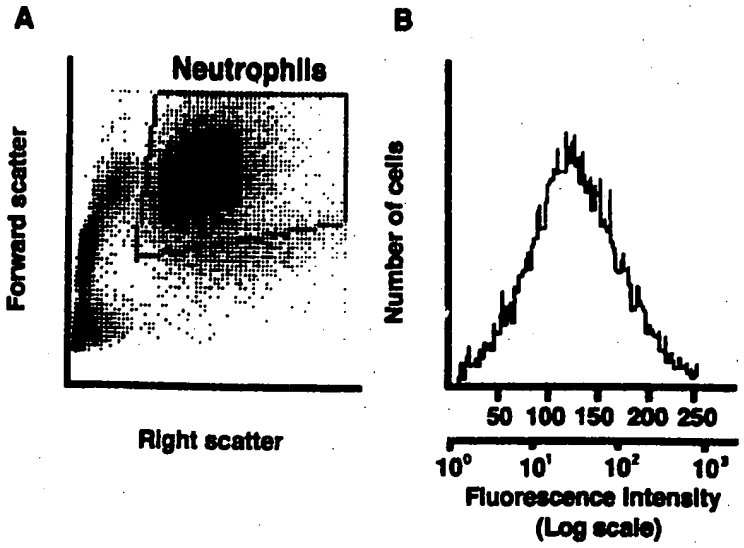

Fig. 1. Representative cytogram **(A)** and histogram **(B)** obtained by flow-cytometric single-cell analysis. Neutrophils can be distinguished from lymphocytes and monocytes by the combination of low-angle forward right-angle scattered laser light. After determination of region of interest of neutrophil population, histogram of fluorescence distribution was constructed. Relative mean intensity of fluorescence is presented as index of neutrophil activation.

riography procedure. Monoclonal antibody (JML-H11)[7] directed against CD11b and fluorescein isothiocyanate–conjugated JML-H11 were reacted with the cell preparation, and the test tube was placed in ice water for 30 minutes. The erythrocytes were hemolyzed with 0.87% NH_4Cl lysing solution. The sample was centrifuged at 100*g* for 6 minutes at 4° C. The pellet was suspended with 5 ml calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) solution containing 0.1% NaN_3 and again was centrifuged at 100*g* for 6 minutes at 4° C. The pellet, dissolved in 2 ml PBSA, was used for flow-cytometric analysis.

A partial modification of the flow-cytometric method developed by Bass et al.[8] was used to measure the oxidative metabolic burst (hydrogen peroxide generation) in neutrophils. Heparinized blood obtained from the coronary sinus was incubated for 15 min in a Dubnoff shaking water bath at 37° C with 5 μmol/L of 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (Eastman Kodak, Rochester, N. Y.) dissolved in ethanol (5 mmol/L). The blood sample was then mixed with 10 μl phorbol myristate acetate with concentration of 25 μg/ml. Immediately after incubation for 20 minutes, the test tube was placed in ice water. Next, 0.5 ml ethylenediamine tetraacetic acid adjusted with calcium- and magnesium-free Dulbecco's PBS containing 5 mmol/L glucose and 0.1% gelatin was added to prevent leukocyte aggregation. The sample was incubated again in a Dubnoff shaking water bath at 37° C for 20 minutes. The test tube was then placed in ice water. Hemolyzation of erythrocytes and subsequent preparation for flow-cytometric analysis were performed as described earlier.

Flow-cytometric analysis was carried out with the Spectrum III (Ortho Diagnostic Systems, Raritan, N. J.). As shown in Fig. 1, neutrophils could be distinguished from lymphocytes and monocytes by the combination of low-angle forward and right-angle scattered laser light, and a cytogram of each cell population could be generated. The region of interest in the neutrophil population was identified by an observer using a computer-aided outlining technique. A histogram of fluorescence distribution was constructed with the fluorescence intensity on the abscissa and the number of neutrophils on the ordinate. Log values were converted to the 256 channels of the log amplifier, which corresponds closely to a 1000-fold scale. The relative mean intensity of fluorescence was obtained from the histogram and expressed as an index of membrane surface expression or of hydrogen peroxide generation in neutrophils.

Measurement of neutrophil elastase. Levels of neutrophil elastase in plasma were measured with an immunoenzymatic method (PMN Elastase kit, Merck Immunoassay, Germany).[9] In brief, plastic tubes coated with antibodies specific to neutrophil elastase formed a complex with the neutrophil elastase–α_1-proteinase inhibitor in the sample. After incubation, the unbound components were removed by decanting the supernatant and washing the tubes. Enzyme-labeled antibodies specific to the α_1-proteinase inhibitor were then added. Alkaline phosphatase was used for labeling, and 4-nitrophenyl phosphate served as the substrate. Substrate hydrolysis was stopped by adding 2 mol/L NaOH solution. The intensity of the color of the reaction mixture was measured as the change in absorbance at 405 nm by a photometer (CL-750, Shimazu, Tokyo) and was proportional to the concentration of the neutrophil elastase–α_1-proteinase inhibitor in the sample. The activity of neutrophil elastase was determined from a calibration curve.

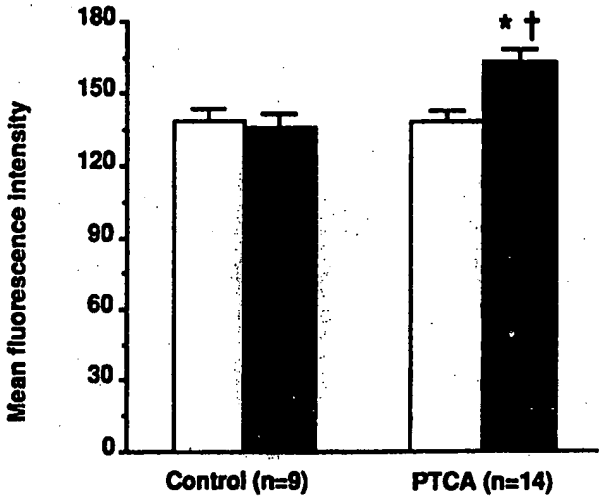

Fig. 2. Changes in mean fluorescence intensity of CD11b in patients undergoing percutaneous transluminal coronary angioplasty *(PTCA)* and in control subjects undergoing coronary arteriography (*open bars*, before procedure; *solid bars*, after procedure). Mean fluorescence intensity after PTCA significantly increased, indicating approximately twofold increase in surface expression of β_2 integrin on neutrophils. *$p < 0.001$ compared with before PTCA; †$p < 0.01$ compared with after coronary arteriography.

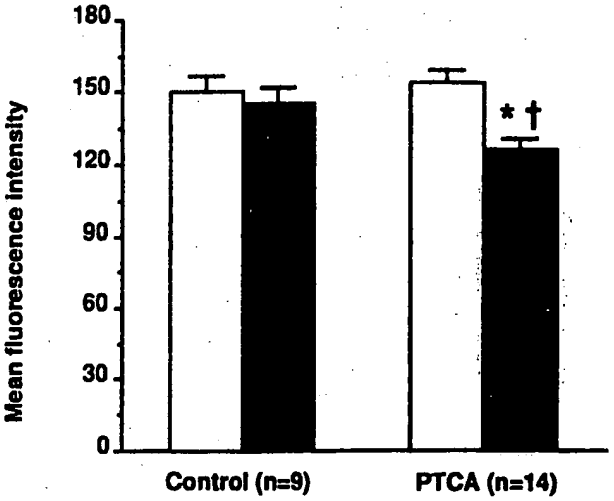

Fig. 3. Changes in mean fluorescence intensity of 2′,7′-dichlorofluorescein in ex vivo phorbol myristate acetate–stimulated neutrophils in patients undergoing percutaneous transluminal coronary angioplasty *(PTCA)* and in control subjects undergoing coronary arteriography (*open bars*, before procedure; *solid bars*, after procedure). Mean fluorescence intensity after PTCA significantly decreased, suggesting that in vivo hydrogen peroxide generation in neutrophils was activated during PTCA. *$p < 0.001$ compared with before PTCA; †$p < 0.05$ compared with after coronary arteriography.

Statistical analysis. Values are presented as means ± SE. Group means were compared by the two-tailed paired or unpaired Student's *t* test. Correlations for the two parameters were obtained by simple linear regression analysis. Differences were considered statistically significant if the *p* value was <0.05.

RESULTS

Percutaneous transluminal coronary angioplasty and control. The clinical profile of the patients who had PTCA is shown in Table I. The procedure was successful in all patients. The degree of coronary stenosis was significantly improved, from 86.5% ± 1.9% to 38.2% ± 1.7% ($p < 0.0001$). Balloon inflation time was 188.6 ± 20.0 (range 90 to 360) seconds. During balloon inflation, six patients had ST-segment elevation; four had ST-segment depression; and four showed no change in the ST segment. Of the patients with a ST-segment change, eight had chest pain. However, no serious complications leading to progression or recurrence of myocardial infarction occurred. The amount of contrast medium used during PTCA procedure was 162.9 ± 9.5 ml.

All patients in the control group had coronary artery disease: one patient had three-vessel disease; five patients had one-vessel disease; and three patients had mild coronary artery stenosis of <50%. The amount of contrast medium used during coronary arteriography was 146.1 ± 8.9 ml, which was not significantly different from the amount used during PTCA.

Surface expression of CD11b on neutrophils. Fig. 2 shows changes in the mean fluorescence intensity of CD11b in patients who underwent coronary arteriography or PTCA. In the 9 control subjects who underwent routine coronary arteriography, no change in mean fluorescence intensity was observed in the coronary sinus (from 138.9 ± 4.7 to 136.2 ± 5.8; *p* not significant [NS]). In the 14 patients with PTCA, mean fluorescence intensity in the coronary sinus increased significantly after PTCA (from 138.1 ± 4.2 to 162.5 ± 5.0, $p < 0.001$). The mean fluorescence intensities after PTCA were significantly higher than those after coronary arteriography (PTCA 162.5 ± 5.0 vs control 136.2 ± 5.8; $p < 0.01$). There was modest correlation between the changes in mean fluorescence intensity of CD11b and inflation time during PTCA, with $r = 0.57$ ($p < 0.05$) but no correlation with the amount of contrast medium or balloon catheter size used during PTCA.

Generation of hydrogen peroxide in neutrophils. Fig. 3 shows changes in mean fluorescence intensity of 2′,7′-dichlorofluorescein in ex vivo PMA-stimulated neutrophils in the 9 control subjects and in the 14

patients undergoing PTCA. Mean fluorescence intensity in the coronary sinus did not change after coronary arteriography (from 150.5 ± 6.7 to 145.1 ± 6.5; p NS), whereas mean fluorescence intensity after PTCA significantly decreased (from 153.7 ± 5.4 to 126.4 ± 4.5; $p < 0.001$). Mean fluorescence intensity after PTCA was significantly lower than that after coronary arteriography (PTCA 126.4 ± 4.5 vs control 145.1 ± 6.5; $p < 0.05$). There was good correlation between the changes in mean fluorescence intensity of 2',7'-dichlorofluorescein and inflation time during PTCA, with $r = 0.64$ ($p < 0.05$), but no correlation were observed with the amount of contrast medium or balloon catheter size used during PTCA procedures.

Release of neutrophil elastase. Fig. 4 shows changes in neutrophil elastase obtained from the coronary sinus in the control subjects and in the patients undergoing PTCA. Neutrophil elastase in the coronary sinus did not change after coronary arteriography (from 104.1 ± 6.1 to 109.5 ± 6.8 μg/L; $n = 9$; p = NS), whereas the elastase in the coronary sinus increased significantly after PTCA (from 109.7 ± 5.7 to 187.6 ± 10.9 μg/L; $n = 10$; $p < 0.001$). There were no correlations between the changes in plasma elastase and inflation time, amount of contrast medium, or balloon catheter size used during PTCA.

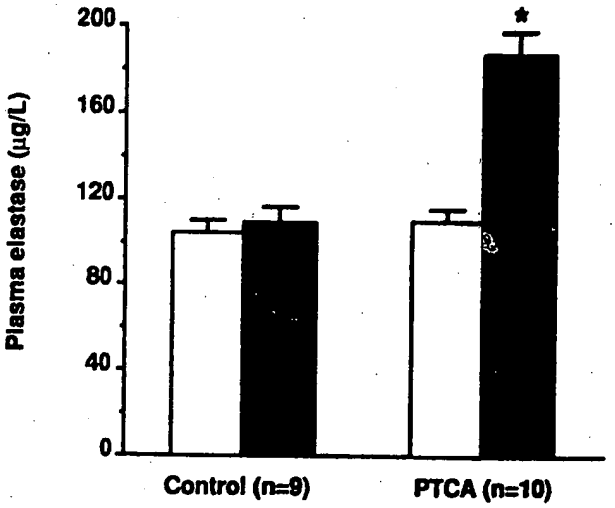

Fig. 4. Changes in neutrophil elastase in patients undergoing percutaneous transluminal coronary angioplasty and control subjects undergoing coronary arteriography (*open bars*, before procedure; *solid bars*, after procedure). Plasma elastase of neutrophil increased significantly after PTCA. *$p < 0.001$ compared with before PTCA.

DISCUSSION

Although PTCA can be regarded as a clinical model of ischemia–reperfusion, information regarding neutrophil function in this condition is limited. In the present study we demonstrated that PTCA induced a significant increase in membrane surface expression of CD11b (β_2 integrin) and in release of elastase and induced a significant decrease in the generation of hydrogen peroxide in ex vivo PMA-stimulated neutrophils. No such changes in neutrophil function were found in the control subjects, who underwent routine coronary arteriography. These observations provide direct evidence that PTCA can lead to neutrophil activation. Furthermore, significant correlations were found between total inflation time and changes in neutrophil function, such as hydrogen peroxide generation and membrane surface expression. In PTCA, the ischemia and subsequent reperfusion, which is not observed in coronary arteriography, may explain its effect on neutrophil function. It is likely that the initial stimulus for neutrophil activation during PTCA is the mechanical occlusion of the vessel by the balloon. Subsequent reperfusion after balloon deflation would further augment neutrophil activation.

To our knowledge, this study is the first to demonstrate increased membrane surface expression of CD11b after PTCA, indicating an increase in neutrophil activation by approximately twofold. CD11b is an adherence molecule classified as β_2 integrin, the integrin supergene family of adhesion.[7] It is a heterodimeric glycoprotein expressed on the plasma membrane of human neutrophils and mononuclear phagocytes.[10] The monoclonal antobidy CD11b inhibits several neutrophil functions, including the binding of C3bi-opsonized particles, adhesive interactions of neutrophils, spreading on vascular endothelium, and chemotaxis.[11] Studies have shown that localized generation of chemotactic complement activity may be an important means of leukocyte stimulation during ischemia–reperfusion.[12] Studies that used a canine model of myocardial ischemia–reperfusion have demonstrated that the surface expression of CD11b/CD18 (Mac-1) adherence molecules was increased on leukocytes in cardiac lymph[13] and that the administration of anti-Mo1 significantly reduced neutrophil accumulation within the infarct area.[5,6] The increased surface expression of CD11b adherence molecules on endogenous neutrophils after PTCA indicates that this procedure directly influences the adhesive properties of neutrophils and that this effect may be an important step in the subsequent process of ischemic injury.[14]

In the presence of hydrogen peroxide released by neutrophils, nonfluorescent DCFH-DA is trapped intracellularly and oxidized to highly fluorescent

2′,7′-dichlorofluorescein. The intensity of 2′,7′-dichlorofluorescein fluorescence in individual neutrophils can be measured by flow cytometry and thus serves as a parameter of hydrogen peroxide generation in neutrophils. In the present study the mean fluorescence intensities in isolated neutrophils after ex vivo PMA stimulation were significantly decreased after PTCA but remained unchanged after coronary arteriography. Because PMA can be considered to induce maximal activation of hydrogen peroxide generation, the reduced responses to PMA stimulation may be explained by previous in vivo activation of neutrophils during PTCA. These observations are similar to results of a previous study demonstrating that stimulated neutrophils release lesser quantities of superoxide anion during PTCA.[15] Experimental studies have demonstrated that hydrogen peroxide can induce neutrophil adherence to endothelial cells[16] and promote the surface expression of adhesion molecule on the leukocyte and on the endothelium.[17] Therefore there may be a pathogenic link between the surface expression of CD11b and the generation of hydrogen peroxide in patients undergoing PTCA. Furthermore, the respiratory metabolic burst of neutrophils is generally related to the activation of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) oxidase, which generates superoxide anions.[18] The superoxide anion is rapidly converted to hydrogen peroxide via the dismutation of superoxide. Therefore the generation of hydrogen peroxide in neutrophils after PTCA may in part represent the activity of the NADPH oxidase pathway, suggesting an important source of oxygen free-radical generation.

Elastase is released extracellularly from neutrophil azurophil granules and is associated with increases in endothelial adhesiveness, permeability, and tissue edema. In addition, elastase has been shown to inhibit the production of prostacyclin[19] and to enhance the release of platelet activating factor[20] in endothelial cells, leading to further promotion of platelet aggregation. The increased release of neutrophil elastase in patients undergoing PTCA in the present study is consistent with previous data.[15] The present data also may be relevant to the results obtained by Mehta et al.,[21] who observed higher plasma elastase levels in patients with unstable angina or acute myocardial infarction but not in patients with stable angina, suggesting that neutrophil activation may be related to the ongoing coronary events in patients with unstable angina or acute myocardial infarction. Thus elastase may play an important role in the pathophysiologic changes in the vessel wall occurring after PTCA.

Study limitations. We acknowledge several limitations to our study. The difference in fluorescence intensities of CD11b and 2′,7′-dichlorofluorescein after PTCA was an approximately a twofold increase above intensities before PTCA; no changes were found in control subjects, who underwent routine coronary arteriography. This finding may be explained by the much shorter duration of ischemia produced by balloon inflation than that used in experimental models. In addition, activated neutrophils may preferentially trapped in the coronary vasculature so that only a small fraction may have entered into the general circulation, resulting in lower intensities. Invasive procedures such as arterial puncture and coronary artery catheterization also may have provoked neutrophil activation, leading to higher baseline values. Recently, the expression of the adhesion molecule CR3 has been shown to decrease after PTCA and coronary arteriography, largely because of the contrast medium.[22] In the present study, the amount of dye given to patients undergoing PTCA was comparable to that given to patients undergoing coronary arteriography. No correlation was found between changes in neutrophil function and the amount of dye or the balloon catheter size used during the procedure. Thus, increased expression of CD11b after PTCA can be attributed to the brief ischemia–reperfusion rather than to the effect of contrast medium during PTCA. However, on the surface of neutrophil there may be many receptors, some of which are activated by acute ischemia–reperfusion and some by dye. Further investigations are required to clarify how PTCA modifies expression of adhesion molecules and thus leads to neutrophil activation.

Clinical implications. Polymorphonuclear leukocytes have been shown to play an important role in the pathogenesis of coronary vascular and myocardial cell injury.[23] The process of neutrophil-mediated tissue injury is thought to be preceded by neutrophil chemotaxis and migration and by adhesion to the vascular endothelium[24] and the cardiac myocytes[25] associated with a CD11/CD18-dependent mechanism. Mechanisms by which neutrophils are activated during ischemia–reperfusion involve the spontaneous trapping of neutrophils,[26] the generation of reactive oxygen free radicals,[27] the increase in neutrophil elastase release,[28] and the generation of arachidonic acid metabolites.[29] These mediators are associated with a complex cascade of cellular interactions involving vascular endothelium and circulating cellular components and contribute to the progression of tissue cell injury. Clinical studies have shown that PTCA induces endothelial dysfunction in

the coronary segments distal to dilated site[30] and regional wall motion abnormalities in diastole.[31] Therefore these alterations after PTCA may be explained in part by the release of mediators through the interaction of neutrophils and endothelial cells.

The development of coronary restenosis after PTCA is a serious and yet unsolved complication. Although the mechanisms of restenosis after successful PTCA are not fully understood, intimal hyperplasia at the intervention site is thought to be important. It has been hypothesized that restenosis is the unique vascular expression of the general wound-healing response.[32] This response to injury is characterized by a sequence of inflammation, granulation, and extracellular matrix remodeling and leads to intimal hyperplasia in the coronary artery after 1 to 4 months. The ischemia–reperfusion produced by PTCA could induce a migration of neutrophils to the injured coronary stenotic site; activated neutrophils release biologically active substances, such as proteolytic enzymes, oxygen free radicals, leukotrienes, and platelet-activating factor that potentiate platelet activity and inhibit endothelial function. The activated platelets would then release substances such as thromboxane, serotonin, adenine nucleotide, and platelet-derived growth factor, leading to vasoconstriction, thrombus formation, and proliferation of vascular smooth muscle.[4, 33, 34] Thus neutrophil activation after PTCA in human beings appears to play an important role in the initial step of the inflammatory phase and then to trigger the pathophysiologic chain reaction eventually resulting in coronary restenosis. However, further studies are required to define fully the mechanisms by which the neutrophil contributes to this complication.

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REFERENCES

1. Ross R. The pathogenesis of atherosclerosis: an update. N Engl J Med 1986;314:488-500.
2. Hansson GK, Jonasson L, Seifert PS, Stemme S. Immune mechanisms in atherosclerosis. Arteriosclerosis 1989;9:567-78.
3. Lucchesi BR, Werns SW, Fantone JC. The role of the neutrophil and free radicals in ischemic myocardial injury. J Mol Cell Cardiol 1989; 21:1241-51.
4. Dinerman JL, Mehta JL. Endothelial, platelet and leukocyte interactions in ischemic heart disease: insights into potential mechanisms and their clinical relevance. J Am Coll Cardiol 1990;16:207-22.
5. Simpson PJ, Todd RF III, Fantone JC, Mickelson JK, Griffin JD, Lucchesi BR. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. J Clin Invest 1988;8:624-9.
6. Dreyer WJ, Michael LH, West SW, Smith CW, Rothlein R, Rossen RD, Anderson DC, Entman ML. Neutrophil accumulation in ischemic canine myocardium: insights into time course, distribution, and mechanism of localization during early reperfusion. Circulation 1991;84:400-11.
7. Buyon JP, Slade SG, Perdue RA, Winchester RJ. CD11b/CD18-dependent homotypic neutrophil aggregation: evidence for discrete adhesiotopes and activation of the molecule. In: Knapp W, Dörken B, Rieber EP, Stein H, Gilks WR, Schmidt RE, Von dem Borne AEGKr, eds. Leukocyte typing IV: white cell differentiation antigens. New York: Oxford, 1989:560-2.
8. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: ε graded response to membrane stimulation. J Immunol 1983;130:1910-7.
9. Neumann S, Gunzer G, Hennrich N, Lang H. PMN-elastase assay: enzyme immunoassay for human polymorphonuclear elastase in complex with α_1-proteinase inhibitor. J Clin Chem Clin Biochem 1984;22:693-7.
10. Todd RF III, Nadler LM, Schlossman SF. Antigens on human monocytes identified by monoclonal antibodies. J Immunol 1981;126:1435-42.
11. Anderson DC, Miller LJ, Schmalsteig FC, Rothlein R, Springer TA. Contribution of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit-specific monoclonal antibodies. J Immunol 1986;137:15-27.
12. Rossen RD, Swain JL, Michael LH, Weakley S, Giannini E, Entman ML. Selective accumulation of the first component of complement and leukocytes in ischemic canine heart muscle: a possible initiator of an extra myocardial mechanism of ischemic injury. Circ Res 1985;57:119-30.
13. Dreyer WJ, Smith CW, Michael LH, Rossen RD, Hughes BJ, Entman ML, Anderson DC. Canine neutrophil activation by cardiac lymph obtained during reperfusion of ischemic myocardium. Circ Res 1989; 65:1751-62.
14. Entman ML, Michael LH, Rossen RD, Dreyer WJ, Anderson DC, Taylor AA, Smith CW. Inflammation in the course of early myocardial ischemia. FASEB J 1991;5:2529-37.
15. De Servi S, Mazzone A, Ricevuti G, Fioravanti A, Bramucci E, Angoli L, Stefano G, Specchia G. Granulocyte activation after coronary angioplasty in humans. Circulation 1990;82:140-6.
16. Lewis MS, Whatley RE, Cain P, McIntyre TM, Prescott SM, Zimmermann GA. Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. J Clin Invest 1988;82:2045-55.
17. Gasic AC, McGuire G, Krater S, Farhood AI, Goldstein MA, Smith CW, Entman ML, Taylor AA. Hydrogen peroxide pretreatment of perfused canine vessels induces ICAM-1 and CD18-dependent neutrophil adherence. Circulation 1991; 84:2154-66.
18. McCord JM. Oxygen-derived free radicals in post-ischemic tissue injury. N Engl J Med 1985;312:159-63.
19. Weksler BB, Jaffe EA, Brower MS, Cole OF. Human leukocyte cathepsin G and elastase specifically suppress thrombin-induced prostacyclin production in human endothelial cells. Blood 1989;74:1627-34.
20. Camussi G, Tetta C, Bussolino F, Baglioni C. Synthesis and release of platelet activating factor is inhibited by plasma α-1 proteinase inhibitor or α-1 antichymotrypsin and is stimulated by proteinases. J Exp Med 1988;168:1293-306.
21. Mehta J, Dinerman J, Mehta P, Saldeen TGP, Lawson D, Donnelly WH, Wallin R. Neutrophil function in ischemic heart disease. Circulation 1989;79:549-56.
22. Feldoman L, Chollet-Martin S, Himbert D, Juliard JM, Pasquier C, Elbim C, Steg PG. Does PTCA really activated granulocytes? role of contrast media [Abstract]. Circulation 1993; 88:I-338.
23. Mehta JL, Nichols WW, Mehta P. Neutrophils as potential participants in acute myocardial ischemia: relevance to reperfusion. J Am Coll Cardiol 1988;11:1309-16.
24. Shappel SB, Toman BC, Anderson DC, Taylor AA, Entman ML, Smith CW. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. J Immunol 1990;144:2702-11.
25. Entman ML, Youker K, Shappel SB, Siegel C, Rothlein R, Dreyer WJ, Schmalstieg FC, Smith CW. Neutrophil adherence to isolated adult canine myocytes: evidence for a CD18-dependent mechanism. J Clin Invest 1990;85:1497-506.
26. Engler RL, Schmid-Schonbein GW, Parelec RS. Leukocyte capillary

plugging in myocardial ischemia and reperfusion in the dog. Am J Pathol 1983;111:98-111.
27. Lucchesi BR. Role of neutrophils in ischemic heart disease: pathophysiologic role in myocardial ischemia and coronary artery reperfusion. In: Mehta JL, ed. Thrombosis and platelets in myocardial ischemia. Philadelphia: FA Davis, 1987:35-48.
28. Weissmann G, Smolen JE, Korchak HM. Release of inflammatory mediators from stimulated neutrophils. N Engl J Med 1980;303:27-34.
29. Mullane KM, Salmon JA, Kraemer R. Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury. Fed Proc 1987;46:2422-33.
30. El-Tamimi H, Davies GJ, Crea F, Maseri A. Response of human coronary arteries to acetylcholine after injury by coronary angioplasty. J Am Coll Cardiol 1993;21:1152-7.
31. Wijns W, Serruys PW, Slager CJ, Grimm J, Krayenbuehl HP, Hugenholtz PG, Hess OM. Effect of coronary occlusion during percutaneous transluminal angioplasty in humans on left ventricular chamber stiffness and regional diastolic pressure-radius relations. J Am Coll Cardiol 1986;7:455-63
32. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J Am Coll Cardiol 1991;17:758-69.
33. Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell 1986;46:155-69.
34. Walker LN, Bowen-Pope DF, Ross R, Reidy MA. Production of platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. Proc Natl Acad Sci U S A 1986;83:7311-15.

Suppression of hyperventilation-induced attacks with infusion of B-type (brain) natriuretic peptide in patients with variant angina

B-type (brain) natriuretic peptide (BNP) forms a peptide family with A-type (atrial) natriuretic peptide (ANP), which is involved in the regulation of blood pressure and fluid volume. We have demonstrated that BNP is a novel cardiac hormone secreted predominantly from the ventricle and that plasma levels of BNP markedly increase in proportion to the severity of congestive heart failure. Spasm of a major coronary artery (coronary spasm) is the cause of variant angina and can be induced by hyperventilation. We examined whether BNP infusion suppresses coronary spasm in patients with variant angina. The effect of BNP infusion on anginal attacks induced by hyperventilation was studied in 11 patients with variant angina in whom the attacks were reproducibly induced by hyperventilation. This study was performed in the early morning on 3 consecutive days. Fourteen minutes after infusion of BNP was begun (day 2, 0.05 μg/kg/min) or saline (days 1 and 3), hyperventilation was started and continued for 6 minutes. Anginal attacks were induced in all 11 patients by hyperventilation on days 1 and 3, respectively. Anginal attacks were not induced in any patient on day 2 (BNP infusion). Fourteen minutes after BNP infusion was begun, plasma BNP levels increased from 23.7 ± 6.7 pg/ml to peak levels of 2591 ± 255 pg/ml ($p < 0.01$) and plasma ANP levels increased from 28.9 ± 7.5 pg/ml to peak levels of 69.2 ± 13.2 pg/ml. Five minutes after BNP infusion was finished, plasma levels of cyclic guanosine monophosphate (cGMP) increased from 20.3 ± 7.4 pg/ml to peak levels of 63.5 ± 13.7 pg/ml ($p < 0.01$). The plasma levels of ANP, BNP, and cGMP did not change after saline infusion and hyperventilation on days 1 and 3, respectively. We conclude that BNP infusion suppresses anginal attacks induced by hyperventilation in patients with variant angina, and that cGMP is related to the mechanism of suppression of the attacks. (Am Heart J 1994;128:1098-104.)

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Expression of Polymorphonuclear Leukocyte Adhesion Molecules and Its Clinical Significance in Patients Treated With Percutaneous Transluminal Coronary Angioplasty

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Objectives. This study evaluated the role of neutrophil adhesion molecules LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) in patients undergoing percutaneous transluminal coronary angioplasty (PTCA).

Background. Several recent studies have suggested that cell adhesion molecules on both neutrophils and vascular endothelial cells play an important role in the process of tissue inflammation.

Methods. Thirty-eight patients (30 men, 8 women; mean [±SE] age 56 ± 5 years, range 38 to 76) with single-vessel coronary artery disease of the left anterior descending artery underwent coronary angioplasty. Peripheral blood was sampled at baseline before, immediately after and 12, 24, 48 and 144 h after PTCA. The expression of CD18, CD11a, CD11b and CD11c on the surface of polymorphonuclear leukocytes was examined by flow cytometry with monoclonal antibodies.

Results. In patients without subsequent restenosis, there was no change in mean channel fluorescence intensity (MFI) of CD18 at each sampling time. However, in the patients with restenosis, the MFI of CD18 significantly increased at 48 h after PTCA (from 57 ± 6 to 73 ± 8, p = 0.0008). The MFI of CD11b increased slightly at 48 h after PTCA in patients without restenosis (from 584 ± 121 to 735 ± 114, p = 0.037). In patients with restenosis, the MFI of CD11b was slightly increased at 24 h after PTCA (from 586 ± 122 to 768 ± 214, p = 0.018) and significantly increased at 48 h after PTCA (to 1,534 ± 268, p = 0.0006). The expression of CD11a and CD11c did not change at any sampling points after PTCA in either of the two patient groups. Percent change in the expression of CD18 at 48 h after PTCA (from baseline) and that of CD11b were correlated (r = 0.73, p = 0.0008) in patients with restenosis.

Conclusions. Inflammatory stimuli within the coronary vessels associated with coronary angioplasty may upregulate Mac-1 expression on the surface of polymorphonuclear leukocytes. This process may be more marked in patients who experience later restenosis. Thus, activation of neutrophil adhesion molecule Mac-1 at 48 h after PTCA may have value as a predictor of subsequent restenosis.

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Polymorphonuclear leukocytes migrate across the vascular endothelium into the extravascular space in response to tissue injury and inflammation (1). Leukocyte adhesion to vascular endothelial cells is an important step in this process (2). It has been shown (3,4) that this adhesion is mediated by adhesion molecules, which are expressed on the cell surface. They induce various cell–cell interactions as receptor–ligand relations (3,4).

The neutrophil adhesion molecules include a family of heterodimic glycoproteins (called beta$_2$-integrins) possessing a common beta-subunit of CD18 associated noncovalently with separate alpha-subunits of CD11a, CD11b and CD11c, and designated as LFA-1, Mac-1 and p150,95, respectively (5–7). The adhesion molecules normally exist on the surface of neutrophils. However, inflammatory stimuli produce an increase in the cell surface expression of these molecules (8,9). Endothelial cell surface molecules, including C3bi, derived from the activation of the complement system, and intercellular adhesion molecule-1 (ICAM-1), interact with neutrophil CD18 adhesion-promoting receptor (5–7). The neutrophils adhere to endothelial cells and when activated can release a variety of mediators capable of promoting tissue injury.

Percutaneous transluminal coronary angioplasty (PTCA) is an established treatment for patients with coronary artery disease. Although PTCA has a high initial success rate, restenosis remains a major problem limiting the long-term efficacy of the procedure (10–12). The mechanism of restenosis has not been fully understood. Several investigators (13–15) have suggested that neutrophils might play a part in the mechanism of this restenosis. Coronary angioplasty induces neutrophil activation, which results in the release of various inflammatory mediators that may potentiate development of smooth muscle cell proliferation and resulting restenosis (13–15).

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Acronyms and Abbreviations

ICAM-1	=	intercellular adhesion molecule-1
IL-1	=	interleukin-1
MFI	=	mean channel fluorescence intensity
PTCA	=	percutaneous transluminal coronary angioplasty
TNF-alpha	=	tumor necrosis factor-alpha

The present study sought to determine the role of leukocyte adhesion molecules LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) in patients undergoing PTCA.

Methods

Patients. Thirty-eight patients (30 men, 8 women; mean [±SEM] age 56 ± 5 years, range 38 to 76) who underwent initial elective PTCA were enrolled in the study. They all had isolated atherosclerotic coronary artery disease (≥75% diameter stenosis) of the proximal left anterior descending artery. All patients had clinically stable angina pectoris without previous myocardial infarction and had evidence of ischemia evaluated by exercise stress electrocardiography and thallium-201 myocardial scintigraphy. All patients received the standard medication for angina, including 40 mg of isosorbide dinitrate, 40 mg of nifedipine, 81 mg of aspirin and 75 mg of dipyridamole daily, and none of these drugs was discontinued before PTCA. Exclusion criteria included receipt of other cardioactive drugs and the presence of other cardiac or noncardiac complications that could affect our analysis. Seven healthy volunteers (five men, two women; mean age 49 ± 7 years, range 32 to 72) were also studied as control subjects. The study protocol was approved by the Dokkyo University Institutional Review Board, and written informed consent was obtained from each patient.

Percutaneous transluminal coronary angioplasty. Coronary angioplasty was performed using the standard Judkins technique and a movable guide wire system through the femoral artery. An 8F guiding catheter (USCI, Inc.) was positioned in the coronary ostium, and control coronary angiograms were obtained. All patients received premedication with 5,000 U of intravenous heparin and 0.1 mg of intracoronary nitroglycerin before angiography. The dilation procedure was performed with multiple balloon inflations using steerable nonperfusion balloon dilation catheters (USCI, Inc.) ranging in diameter from 2.5 to 3.5 mm when inflated. Optimal balloon sizes were chosen on the basis of estimates of reference diameter of normal segments adjacent to the lesion. Each balloon inflation was maintained for 90 s at a pressure range from 6 to 12 atm. A nonionic iodinated contrast agent was used during the procedure (Iopamidole, Schering AG) in all patients. After PTCA, all patients received 500 U/h of intravenous heparin for 24 h. Any oral medications were not changed until follow-up angiography. Follow-up angiography was recommended to all patients at 6 months after PTCA and was performed earlier if there were clinical indications. Coronary lesions were assessed angiographically before and immediately after PTCA and at follow-up coronary angiography. The method used, caliper measurements in multiple projections, adequately demonstrated the lesions. Primary success of angioplasty was defined as a 20% increase in lumen diameter and a residual diameter stenosis <50%. For purposes of follow-up angiography, restenosis was defined as >50% diameter stenosis.

Flow cytometric analysis of leukocyte adhesion molecules. In each patient, peripheral blood was sampled at baseline before, immediately after and 12, 24, 48 and 144 h after PTCA. Blood samples were also obtained from the healthy volunteers. The expression of CD18, CD11a, CD11b and CD11c on the surface of polymorphonuclear leukocytes was analyzed by flow cytometry with monoclonal antibodies. Blood was immediately collected in a tube containing acid citrate dextrose (ACD). Two-color immunofluorescence staining (16) was performed using fluorescein isothiocyanate (FITC) (17) conjugated anti-CD18 (IOT18, Immunotech, Inc.) and anti-CD11a (IOT16, Immunotech, Inc.) and phycoerythrin (PE) (18) conjugated anti-CD11b (Lew 15, Becton Dickinson) and anti-CD11c (LewM5, Becton Dickinson). After hemolysis was completed by the lysing solution, cells were fixed in a paraformaldehyde solution with phosphate-buffered saline (PBS). The staining process was performed according to the National Committee for Clinical Laboratory Standards guidelines for flow cytometry (19). Two-color flow cytometric analysis was then performed using a FACScan laser flow cytometry system (Becton Dickinson) within 2 h. We collected the data from 10,000 cells/test and analyzed the scatter signals and fluorescence intensity. The light-scattering properties projected on a scattergram could distinguish the polymorphonuclear leukocyte cluster from other leukocyte clusters (20). Fluorescence intensity was expressed on a cytohistogram where the region of interest was limited to the polymorphonuclear leukocyte cluster for CD18, CD11a, CD11b, and CD11c each. Mean channel fluorescence intensity (MFI) (21) was calculated as the index of the expression of each adhesion molecule.

Data analysis. Coronary angiograms were assessed by two independent observers (T.I., Y.S.). Each observer measured the severity of stenosis using the same technique, and the mean value was recorded. There was a 1.8 ± 5.7% difference between the two observers on 30 randomly selected lesions, excluding those of the study patients. Serial changes in the MFI of CD18, CD11a, CD11b and CD11c were each analyzed using repeated measures analysis of variance in patients with and without restenosis for intragroup and intergroup comparisons. Comparisons of the values between the two patient groups and the control subjects were performed using one-way analysis of variance. The chi-square test or unpaired *t* test was used for intergroup comparisons of baseline characteristics. Correlation between percent increase in the MFI of CD18 and that of CD11b was determined with linear regression in each patient group. To predict the occurrence of restenosis, the sensitivity of increases in the MFI of CD18 or CD11b, or both, was

Table 1. Clinical Characteristics of 38 Study Patients

	Pts Without Restenosis (n = 24)	Pts With Restenosis (n = 14)	p Value
Age	55 ± 4	57 ± 3	0.366
Men/women (no.)	19/5	11/3	0.284
Leukocyte count (/μl)	6,760 ± 360	6,270 ± 290	0.226
Platelet count (×10⁴/μl)	28.5 ± 4.6	30.2 ± 5.8	0.183
PT (s)	11.2 ± 0.3	11.3 ± 0.2	0.676
APTT (s)	31.6 ± 0.6	31.9 ± 0.5	0.720
Coronary risk factors			
Cigarette smoking	21 (88%)	12 (86%)	0.624
Family history	7 (29%)	4 (28%)	0.641
Diabetes mellitus	4 (17%)	2 (14%)	0.528
Systemic hypertension	7 (29%)	4 (28%)	0.725
Total cholesterol (mg/dl)			
Before PTCA	207 ± 9	214 ± 12	0.465
At follow-up study	197 ± 8	199 ± 14	0.673
HDL cholesterol (mg/dl)			
Before PTCA	37 ± 2	36 ± 2	0.322
At follow-up study	39 ± 4	39 ± 3	0.864
Taking lipid-lowering drugs	4 (17%)	2 (14%)	0.464

Data presented are mean ± SE or number (%) of patients (Pts). APTT = activated partial thromboplastin time; HDL = high density lipoprotein; PT = prothrombin time; PTCA = percutaneous transluminal coronary angioplasty.

determined as true positive/(true positive + false negative), specificity as true negative/(true negative + false positive), positive predictive values as true positive/(true positive + false positive) and negative predictive values as true negative/(true negative + false negative). Results are expressed as mean value ± SE; $p < 0.05$ was considered significant.

Results

Results of angioplasty. Balloon inflations ranged from three to eight (mean 4.2 ± 0.3), and PTCA was initially successful in all patients. No patient experienced either abrupt coronary occlusion or major coronary dissection. However, restenosis was seen in 14 patients at follow-up angiography. Comparison of 24 patients without restenosis and 14 with restenosis revealed no significant differences in age, gender, leukocyte counts, platelet counts and blood coagulation activity before PTCA. There were also no significant differences among the patients with regard to coronary risk factors, such as smoking habits, family history, diabetes mellitus, hypertension and hyperlipidemia (Table 1). Characteristics of coronary lesions, including reference diameter, American College of Cardiology/American Heart Association classification of lesion type, American Heart Association classification of lesion location and severity of stenosis before and immediately after PTCA, were similar in patients with and without restenosis. Furthermore, there were no significant differences between patients with and without restenosis with regard to the angioplasty procedure itself (balloon size, number of inflations, inflation pressures, duration of inflations, duration of the procedure and amount of contrast medium used). In patients with restenosis, time to follow-up study after PTCA was 4.8 ± 1.1 months, possibly indicating the interval after PTCA when restenosis was first clinically demonstrable, whereas it was 6.6 ± 0.4 months ($p = 0.045$) in patients without restenosis (Table 2).

Expression of leukocyte adhesion molecules. Serial changes in adhesion molecule expression in patients with and without restenosis compared with control values are shown in Figure 1. The MFI for CD18 at each sampling time did not change immediately or 12, 24 or 48 h after PTCA from baseline values in patients without restenosis. However, in patients with restenosis, it significantly increased at 48 h after PTCA (from 57 ± 6 to 73 ± 8, $p = 0.0008$). The MFI for CD11b increased slightly at 48 h after PTCA even in patients without restenosis (from 584 ± 121 to 735 ± 114, $p = 0.037$). In the restenosis group, the MFI for CD11b increased slightly at 24 h after PTCA (from 586 ± 122 to 768 ± 214, $p = 0.018$) and increased significantly at 48 h after PTCA (to 1,534 ± 268, $p = 0.0006$). The expression of CD11a and CD11c in both patient groups did not change at any sampling time. A comparison of patients with and without restenosis at each sampling time reveals that there were no significant differences in MFI values, except that for CD11b at 48 h after PTCA, which was slightly higher ($p = 0.021$) in patients with restenosis. A comparison of each patient group versus control values shows no significant difference, except for the MFI for CD18 ($p = 0.046$) and CD11b ($p = 0.009$) at 48 h after PTCA, which were higher in patients with restenosis.

Figure 2 shows a correlation between the percent change in expression at 48 h after PTCA from the baseline value of CD18 and CD11b in each patient. Both changes were correlated in patients with ($r = 0.73$, $p = 0.0008$) but not in those without ($r = 0.17$, $p = 0.382$) restenosis. This relation also showed that the increase in expression of both CD18 and CD11b at 48 h

Table 2. Profiles of Coronary Lesions and Angioplasty Procedure in 38 Study Patients

	Pts Without Restenosis (n = 24)	Pts With Restenosis (n = 14)	p Value
Reference diameter (mm)	2.82 ± 0.07	2.79 ± 0.06	0.322
ACC/AHA lesion type (A/B)	14/10	8/6	0.283
AHA lesion location (seg 6/seg 7)	10/14	7/7	0.128
% diameter stenosis			
Pre-PTCA	86.9 ± 3.4	85.2 ± 2.8	0.267
Post-PTCA	14.0 ± 2.5	15.6 ± 3.2	0.324
Follow-up study	36.5 ± 4.2	64.8 ± 8.6	0.0003
Final balloon size (mm)	2.91 ± 0.07	2.89 ± 0.08	0.362
No. of inflation trials (times)	4.1 ± 0.3	4.2 ± 0.4	0.456
Peak inflation pressure (atm)	11.2 ± 0.3	11.1 ± 0.2	0.867
Duration of total inflations (s)	368 ± 27	382 ± 36	0.572
Duration of all procedures (min)	56 ± 3	59 ± 3	0.120
Amount of contrast medium (ml)	148 ± 7	152 ± 9	0.261
Follow-up term (mo)	6.6 ± 0.4	4.8 ± 1.1	0.045

Data presented are mean value ± SE or number of lesions. ACC = American College of Cardiology; AHA = American Heart Association; seg = segment; other abbreviations as in Table 1.

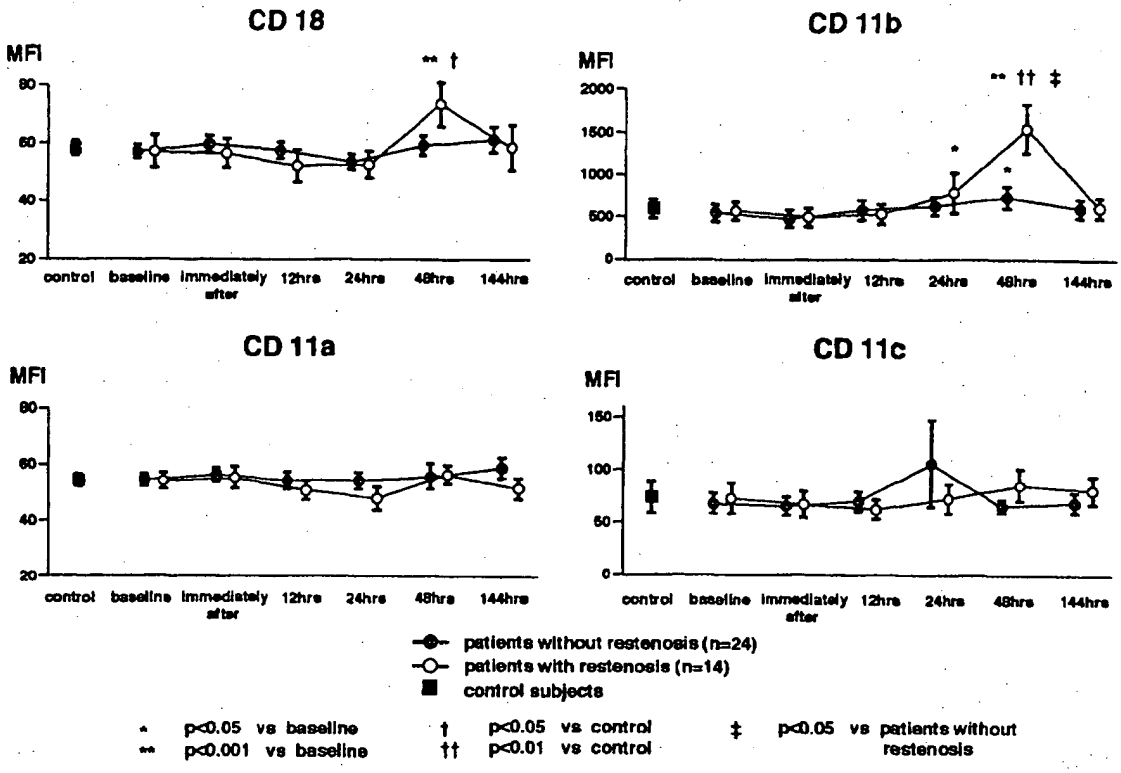
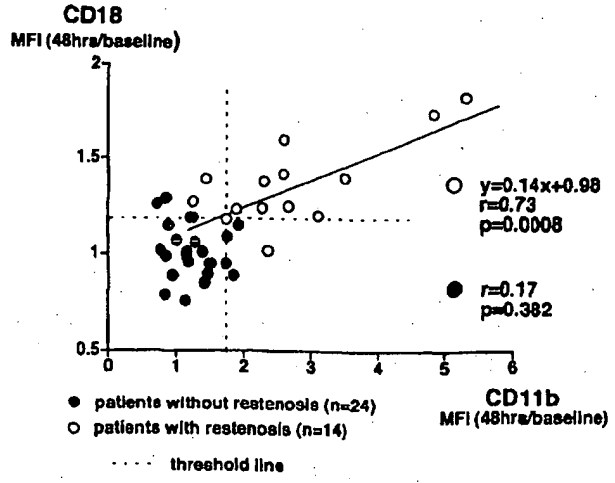


Figure 1. Serial changes of each leukocyte adhesion molecule expression evaluated separately in patients with and without restenosis compared with control values. See Results for details.

was greater in patients with restenosis. In our analysis of percent change in CD18 at 48 h after PTCA, a threshold value of 1.2 had a sensitivity of 86%, a specificity of 83%, a positive predictive value of 75% and a negative predictive value of 91%. For analysis of the percent change in CD11b, a threshold value of 1.7 had a sensitivity of 79%, a specificity of 88%, a positive predictive value of 79% and a negative predictive value of 88%. Furthermore, for both a >1.2-fold increase in expression of CD18 and a >1.7-fold increase in that of CD11b, the sensitivity, specificity and positive and negative predictive values were 71%, 100%, 100% and 86%, respectively.

Figure 2. Correlation between percent change in adhesion molecule expression at 48 h after PTCA from baseline value of CD18 and CD11b in each patient. See Results for details.



Discussion

In our study, only patients with single-vessel coronary artery disease of the proximal left anterior descending artery were selected for inclusion. All patients had stable angina and no previous myocardial infarction, and all were given identical medications during the postprocedural period. We demonstrated that the expression of CD18 and CD11b on the surface of polymorphonuclear leukocytes sampled from peripheral blood increased at 48 h after PTCA, whereas expression of CD11a and CD11c did not. Increases in the expression of CD18 and CD11b at 48 h after PTCA were more prominent in patients with restenosis group despite the finding that baseline characteristics (including invasive procedure, coronary risk factors and leukocyte count) were similar between patients with and without restenosis. Furthermore, the relation between both increases in CD18 and CD11b shown in the restenosis group indicates that the increase in CD18 expression might correspond to the increase in Mac-1. These results suggest that the repeated balloon inflations during PTCA resulted in an upregulation of Mac-1 on the surface of

polymorphonuclear leukocytes at 48 h after PTCA that might be related to the development of restenosis. Moreover, the upregulation of Mac-1 at 48 h after PTCA had a high predictive value for subsequent restenosis.

Expression of neutrophil adhesion molecules as a marker for inflammation. It is widely recognized that adhesion molecules CD18, CD11a, CD11b and CD11c are expressed on the surface of circulatory neutrophils even under normal conditions and can be upregulated severalfold by inflammatory stimuli using various cytokines, including tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1) or endotoxins (8,9). In our study, no significant differences were seen in MFI values of the adhesion molecules in patients with and without restenosis at any time point or in these patients compared with control subjects, except for the values of CD18 and CD11b at 48 h after PTCA. These values showed large individual variations not only in the patient groups but also among the control subjects. However, serial changes in an individual are suggestive of greater significance, and the findings that the values of CD18 and CD11b increased at 48 h after PTCA in patients with restenosis might be of value.

Recently, neutrophil adhesion molecules LFA-1, Mac-1 or p150,95, alone or in combination, have been postulated to play a role in myocardial reperfusion injury (22–27). Endothelial injury secondary to ischemia and reperfusion promotes neutrophil adherence to the endothelial cell surface of the postcapillary venules. However, these phenomena have been recognized mainly in experimental studies with animals, and the role of adhesion molecules in the human heart remains controversial. Our data suggest that leukocyte adhesion molecules may also have a significant impact on the human heart.

Restenosis after coronary angioplasty. Restenosis is the major limitation to the long-term success of PTCA. Restenosis occurs within several months of the procedure in approximately one-third of patients (10–12). The pathophysiology of restenosis has been controversial, but several histopathologic studies indicate that intimal proliferation of smooth muscle cells is a major mechanism (28). Traumatic injury of the vessel wall during angioplasty probably triggers a series of cellular and subcellular events that may ultimately lead to myointimal proliferation and restenosis. Although the exact mechanism by which this occurs is unknown, several factors may enhance smooth muscle cell growth and therefore play a role in the development of restenosis. These include platelet deposition, mechanical stretching of the media; inflammation of the vessel wall; the activity of growth factors, such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF); vasoactive substances, such as serotonin and thromboxane A_2; wall shear stress; alterations in vessel geometry; and other, still unknown factors (29,30). Leukocyte function has recently been singled out for its role in smooth muscle cell proliferation. In addition to production of various growth factors by macrophages (31), activated neutrophils have been also implicated in platelet activation and smooth muscle cell proliferation (13–15,32).

Role of neutrophils in mechanism of restenosis. Coronary angioplasty induces neutrophil activation, which results in the release of a variety of inflammatory mediators, for example, granulocyte proteases, such as elastase, and superoxide anion (14,15). The release of proteolytic enzymes and the generation of oxygen free radicals may aggravate the endothelial damage and further stimulate platelets. This process has potential implications in the subsequent development of smooth muscle cell proliferation and resulting restenosis. During the process of neutrophil activation, the adhesion of neutrophils to vascular endothelial cells is an important physiologic interaction. Thus, it is suggested that the adhesion molecules on the surface of both neutrophils and endothelial cells may play a role in the mechanisms of restenosis.

Two possible mechanisms explain how PTCA induces the upregulation of Mac-1: 1) This process may result from ischemia–reperfusion cycles induced by repeated balloon inflations; 2) the traumatic vascular injury by balloon inflation may result in this upregulation. Although neutrophil activation may be triggered by 15 min of ischemia followed by reperfusion (22), it is not known whether repeated but shorter bouts of ischemia followed by restoration of flow, as occurs during PTCA, could have the same effects. Mazzone et al. (33) observed increased expression of adhesion molecules CD18 and CD11b on the surface of neutrophils taken from the coronary sinus in patients with unstable angina. They speculated that plaque rupture and subsequent inflammation associated with considerable coronary vascular damage in unstable angina might increase the expression of these molecules. In line with their suggestion, the traumatic vascular injury may be the more important factor. In our study, it would appear that inflammatory stimuli within the coronary vessels attributable to coronary angioplasty may upregulate Mac-1 expression on the surface of polymorphonuclear leukocytes. Therefore, the upregulation of Mac-1 may be an indicator of injury or inflammation within the vessel.

Ikeda et al. (34) observed that neutrophil surface expression of CD11b in the coronary sinus blood increased immediately after PTCA. In that report, however, the expression was evaluated immediately after PTCA only, whereas our study showed an increase at 48 h after PTCA. We cannot explain why upregulation occurred only at 48 h after the procedure. We believe that the magnitude of inflammatory stimuli may be related to the duration of leukocyte activation and that ex vivo stimuli using cytokines or endotoxins produced immediate upregulation of Mac-1 on the surface of leukocytes. In contrast, Tanaka et al. (35) observed the expression of ICAM-1, a counterreceptor of LFA-1 and Mac-1, on the endothelial cell surface of rabbit aorta 48 h after balloon injury. Prescott et al. (32) observed that neutrophils adhered to endothelium at 48 h after inducing leukocyte migration by the implantation of an endotoxin-soaked cotton thread in the adventitia of the rat femoral artery. If the nature of inflammatory stimuli produced by PTCA angioplasty in humans is similar to that seen in those experiments, our results at 48 h may be understandable. In addition, in view of the short life expectancy of the polymor-

phonuclear leukocyte, it is likely that PTCA creates a persistent proinflammatory surface within the artery that can stimulate leukocyte expression of Mac-1 for 2 days or that the section of artery traumatized by the balloon inflations begins to secrete cytokines such as TNF-alpha or IL-1 ~24 to 36 h after the procedure, which can upregulate surface expression of Mac-1. The same cytokines that stimulate expression of the leukocyte adhesion molecules may also stimulate smooth muscle cell proliferation (36).

In contrast, the study by Ikeda et al. (34) did not indicate a direct relation between CD11b expression and the progression of restenosis. To our knowledge, our study is the first to demonstrate that the upregulation of Mac-1 is significantly greater in patients with than without restenosis. Although our study does not necessarily prove that this upregulation has a direct role in the mechanism of restenosis, it does suggest some relation between this finding and the pathogenesis of restenosis. Moreover, our results also suggest that activation of the polymorphonuclear leukocyte Mac-1 at 48 h after PTCA may have value as a predictor of subsequent restenosis.

Potential limitations. Our study has several possible limitations. Although we showed that the observed increase in CD18 and CD11b had high predictive values for the occurrence of restenosis, these data were analyzed only retrospectively. Another prospective trial to validate this prediction should be considered. In our study, coronary lesions were assessed angiographically using only caliper measurements. In light of recent progression of quantitative coronary angiographic analysis, we would need to interpret the relation between leukocyte function and quantitative coronary angiographic data.

In addition, the expression of leukocyte adhesion molecules in our study may have been modified by invasive procedures, such as arterial puncture or coronary artery catheterization. The introduction of infusion materials, such as contrast medium (37) or heparin (38), might also influence leukocyte function. However, there may still be significance in the finding that serial patterns for the expression of CD18 and CD11b were different in patient groups with and without restenosis because these groups were similar in terms of the invasive procedures performed, amount of contrast medium used and dose and duration of heparin.

Clinical implications. Our study demonstrated that the expression of CD18 and CD11b on the surface of polymorphonuclear leukocytes sampled from peripheral blood increased at 48 h after PTCA. Inflammatory stimuli within the coronary vessels, which may be attributable to repeated short-term ischemia–reperfusion cycles or traumatic vascular injury associated with the angioplasty, or both, may upregulate Mac-1 on the surface of polymorphonuclear leukocytes. This process may be more pronounced in patients who experience later restenosis. Thus, the upregulation of polymorphonuclear leukocyte Mac-1 at 48 h after PTCA may have value as a predictor of restenosis.

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References

1. Weiss SJ. Tissue destruction by neutrophil. N Engl J Med 1989;320:365–77.
2. Toothil VJ, Van Mourik JA, Niewenhuis HK, Metzelaar MJ, Pearson JD. Characterization of the enhanced adhesion of neutrophil leukocytes to thrombin-stimulated endothelial cells. J Immunol 1990;145:283–91.
3. Detmers PA, Wright SD. Adhesion-promoting receptors on leukocytes. Curr Opin Immunol 1988;1:10–5.
4. Smith CW, Rothlein R, Hughes BJ, et al. Recognition of an endothelial determinant for CD-18 dependent human neutrophil adherence and transendothelial migration. J Clin Invest 1988;82:1746–56.
5. Sanchez-Madrid F, Nagy JA, Robbins E, Simon P, Springer TA. A human leukocyte differentiation antigen family with distinct α-subunits and a common β-subunit: the leukocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecules. J Exp Med 1983;158:1785–803.
6. Kishimoto TK, O'Connor K, Lee A, Roberts TM, Springer TA. Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell 1987;48: 681–90.
7. Patarroyo M, Prieto J, Rincon J, et al. Leukocyte-cell adhesion: a molecular process fundamental in leukocyte physiology. Immunol Rev 1990;14:67–108.
8. Arnaout NA, Lanier LL, Faller DV. Relative contribution of the leukocyte molecules Mo1, LFA-1, and p150.95 (Lew M5) in adhesion of granulocytes and monocytes to vascular endothelium is tissue- and stimulus-specific. J Cell Physiol 1988;137:305–9.
9. Freuyer DR, Morganroth ML, Todd RF. Surface Mo1 (CD11b/CD18) glycoprotein is upmodulated by neutrophils recruited to sites of inflammation in vivo. Inflammation 1989;13:495–505.
10. Kent KM, Bentivogolio LG, Block PC, et al. Percutaneous transluminal coronary angioplasty: report from the registry of the National Heart, Lung, and Blood Institute. Am J Cardiol 1982;49:2011–20.
11. Holmes DR, Vliestra RE, Smith HC, et al. Restenosis after percutaneous transluminal coronary angioplasty (PTCA): a report from the PTCA registry of the National Heart, Lung, and Blood Institute. Am J Cardiol 1984;53: 53C–5C.
12. Leimgruber PP, Robin GS, Hollman J, et al. Restenosis after successful coronary angioplasty in patients with single-vessel disease. Circulation 1986;73:710–7.
13. Cole CW, Hagen P-O, Lucas JF, et al. Association of polymorphonuclear leukocytes with sites of aortic catheter-induced injury in rabbits. Atherosclerosis 1987;67:229–36.
14. De Servi S, Mazzone A, Ricevuti G, et al. Granulocyte activation after coronary angioplasty in human. Circulation 1990;82:140–6.
15. Ricevuti G, Mazzone A, Pasotti D, De Servi S, Specchia G. Role of granulocytes in endothelial injury in coronary heart disease in humans. Atherosclerosis 1991;91:1–14.
16. Bruhring HJ, Asenbauer B, Katrilaka K, Humel G, Busch FW. Sequential expression of CD34 and CD33 antigens on myeloid colony-forming cells. Eur J Haematol 1989;42:143–9.
17. Hardy RR, Hayakawa K, Kaaijman J, Herzenberg IA. B-cell subpopulations identified by two-color fluorescence analysis. Nature 1982;297:589–91.
18. Oi VT, Glazer AN, Stryer L. Fluorescent phicobiliprotein conjugates for analysis of cells and molecules. J Cell Biol 1982;93:981–6.
19. The National Committee for Clinical Laboratory Standards. Clinical applications of flow cytometry: quality assurance and immunophenotyping of peripheral blood lymphocytes. NCCLS 1992;12:1–76.
20. Stephen HI, Ritterhaus CW, Hearley KW, Struzziero CC, Hoffman RA, Hansen PW. Rapid enumeration of T lymphocytes by a flow-cytometric immunofluorescence method. Clin Chem 1982;28:1905–9.
21. Wells DA, Daigneault-Creech CA, Simrell CR. Effect of iron status on reticulocyte mean channel fluorescence. Am J Clin Pathol 1992;97:130–4.

22. Lucchesi BR. Myocardial ischemia, reperfusion and free radical injury. Am J Cardiol 1990;65:141–231.
23. Simpson PJ, Todd RF, Fantone JC, Mickelson JK, Griffin JD, Lucchesi BR. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. J Clin Invest 1988;81:624–9.
24. Simpson PJ, Todd RF, Mickelson JKJ, et al. Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function. Circulation 1990;81:226–37.
25. Williams FM, Collins PD, Tanniere-Zeller M, Williams TJ. The relationship between neutrophils and increased microvascular permeability in a model of myocardial ischaemia and reperfusion in the rabbit. Br J Pharmacol 1990; 100:729–34.
26. Yamazaki T, Seko Y, Tamatani T, et al. Expression of intercellular adhesion molecule-1 in rat heart with ischemia/reperfusion and limitation of infarct size by treatment with antibodies against cell adhesion molecules. Am J Pathol 1993;143:410–8.
27. Lefer DJ, Shandelya SML, Serrano Jr CV, Becker LC, Kuppusany P, Zweier JL. Cardioprotective actions of a monoclonal antibody against CD-18 in myocardial ischemia-reperfusion injury. Circulation 1993;88(Pt 1):1779–87.
28. Faxon DP, Sanborn TA, Weber VJ, et al. Restenosis following transluminal angioplasty in experimental atherosclerosis. Atherosclerosis 1984;4:189–95.
29. Liu MW, Roubin GS, King III SB. Restenosis after coronary angioplasty: potential biologic determinants and role of intimal hyperplasia. Am J Cardiol 1988;79:1374–87.
30. Karas SPI, Santoian EC, Gravanis MB. Restenosis following coronary angioplasty. Clin Cardiol 1991;14:791–801.
31. Munro JM, Cotran RS. The pathogenesis of atherosclerosis: atherogenesis and inflammation. Lab Invest 1988;58:249–61.
32. Prescott MF, McBride CK, Court M. Development of intimal lesions after leukocyte migration into the vascular wall. Am J Pathol 1989;135:835–46.
33. Mazzone A, De Servi S, Ricevuti G, et al. Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. Circulation 1993;88:358–63.
34. Ikeda H, Nakayama H, Oda T, et al. Neutrophil activation after percutaneous transluminal coronary angioplasty. Am Heart J 1994;128:1091–8.
35. Tanaka H, Sukhova GK, Swanson SJ, et al. Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. Circulation 1993;88(Pt 1):1788–803.
36. Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis: a special case of atherosclerosis progression. Circulation 1992;86 Suppl III:III-47–52.
37. Feldman LJ, Chollet-Martin S, Himbert D, et al. Modulation of the expression of the granulocyte adhesion molecule, CR3, by percutaneous transluminal coronary angioplasty and contrast media. Invest Radiol 1994; 29:313–8.
38. Bazzoni G, Nunez AB, Mascellani G, Bianchini P, Dejana E, del Maschio A. Effect of heparin, dermatan sulfate, and related oligo-derivatives on human polymorphonuclear leukocyte functions. J Lab Clin Med 1993;121:268–75.

Mononuclear Leukocytes Invade Rabbit Arterial Intima During Thickening Formation via CD18- and VLA-4–Dependent Mechanisms and Stimulate Smooth Muscle Migration

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Abstract The role of mononuclear leukocytes for the migration of smooth muscle cells (SMCs) during intimal thickening was investigated in the rabbit model of electrically stimulated carotid artery. The approach was to inhibit leukocyte entry into the arterial intima with antibodies against the adhesion molecules very late activation antigen-4 (VLA-4) and CD11/CD18. In electrically stimulated control rabbits treated either with saline or a nonspecific antibody, all types of granulocytes, monocytes, and lymphocytes migrated across an intact endothelium into the acellular subendothelial space, followed by the movement of SMCs from the media into the intima within 36 hours of applying electrical current. Treatment of the rabbits with monoclonal antibody (mAb) HP1/2 directed toward the α_4 subunit (CD49d) of VLA-4 inhibited mononuclear leukocyte invasion (consisting of monocytes and lymphocytes) by ≈70% compared with the IgG-treated control rabbits and completely abolished the minimal influx of basophils and eosinophils after 36 hours. Neutrophil infiltration, however, remained unaffected by anti-VLA-α_4 treatment. Under these conditions, SMC migration across the internal elastic lamina was reduced by 50%. The use of mAb HP1/2 together with mAb 60.3 (directed to the β_2 chain of CD11/CD18) completely abolished the influx of monocytes, lymphocytes, and all types of granulocytes into the arterial intima. This complete blockade of leukocyte infiltration resulted in a 70% reduction of intimal SMC accumulation. Together with our previous findings excluding neutrophils as stimulators of SMC migration, the present results indicate that mononuclear leukocytes promote lesion development by stimulating SMC migration. (***Circ Res.* 1995;77:1121-1128.**)

Key Words • β_1 and β_2 integrins • arteriosclerosis • monocytes • lymphocytes • granulocytes

The accumulation of monocytes and lymphocytes is a prominent feature in human and experimental atherosclerosis.[1-3] They are assumed to contribute to the initiation and progression of atherosclerotic plaques by secreting a variety of inflammatory mediators, cytokines, and growth factors.[2,4-6] Secretory products of monocytes/macrophages may serve as chemoattractants[7-9] or mitogens[10,11] for vascular SMCs and may hence promote SMC accumulation within the intima during atherogenesis. T lymphocytes, on the other hand, are capable of introducing growth-inhibiting factors for SMCs into the atherosclerotic plaques,[2,12] but they may also indirectly stimulate SMC proliferation and/or migration by macrophage activation.[2] A few reports exist indicating a link between leukocyte migration into the vascular wall and intimal SMC accumulation during experimental intimal thickening. In particular, intimal lesion formation induced by an endotoxin-soaked thread in rat femoral arteries was prevented by inhibiting leukocyte invasion into the vessel wall after treatment with dexamethasone.[13] Likewise, neointimal thickening elicited by balloon catheter injury in rat carotid arteries was reduced after inhibiting the accumulation of CD4-positive mononuclear leukocytes in the intima,[14] suggesting a role of CD4-positive mononuclear cells in mediating intimal SMC accumulation.

Our interest was to study the functional significance of mononuclear leukocyte invasion in SMC migration during experimental intimal thickening. We inhibited mononuclear leukocyte infiltration into the nascent thickening by interfering with leukocyte/endothelial interactions, thus gaining additional insight into the recruitment mechanisms of mononuclear leukocytes. Intimal thickening was induced by repeated transmural ES of the rabbit carotid artery. By use of this model, lesions develop in the presence of a continuous yet functionally and structurally altered endothelium.[15,16] Granulocytes, monocytes, and lymphocytes invade the acellular intimal space within the first 2 days of application of direct electrical current.[16-18] Concomitantly, SMCs start to migrate across the internal elastic lamina from the media toward the intima. We previously demonstrated that the initial invasion of neutrophilic granulocytes was completely abolished by treating the rabbits with an antibody directed toward the common β_2 chain (CD18) of the leukocyte adherence glycoprotein complex CD11/CD18, whereas the influx of mononuclear leukocytes was only partially inhibited (by ≈50%).[17] Under these conditions, SMC migration into the intima was not significantly affected, thus excluding neutrophils as initi-

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Selected Abbreviations and Acronyms

EC = endothelial cell
ES = electrical stimulation (electrically stimulated)
ICAM = intercellular adhesion molecule
mAb = monoclonal antibody
PDGF = platelet-derived growth factor
PMN = polymorphonuclear neutrophil
SMC = smooth muscle cell
VCAM = vascular cell adhesion molecule
VLA = very late activation antigen

ators of the directed movement of SMCs into the intimal compartment.

The purpose of the present study was (1) to suppress the residual CD18-independent portion of the invasion of mononuclear leukocytes and (2) to investigate the effect of the blocked influx of monocytes and lymphocytes on the migratory behavior of SMCs in the initial phase of lesion development. The blocking agent we used was a monoclonal antibody that binds to the α_4 chain (CD49d) of VLA-4 (CD49d/CD29). VLA-4 is a member of the β_1-integrin subfamily and is expressed on resting monocytes and lymphocytes[19,20] and on basophils and eosinophils[21-23] but not on neutrophils.[21] It participates not only in cell-matrix interaction but also in the recruitment of these cells from the bloodstream to areas of infection and inflammation.[19,20,23,24] Its counterreceptor on endothelial cells is the cytokine-induced ligand VCAM-1.[25]

Our adhesion-blocking experiments provide evidence that the initial recruitment of monocytes and lymphocytes during the development of fibromuscular intimal thickening is mediated by VLA-4–dependent as well as CD18-dependent pathways. Moreover, the blockade of mononuclear leukocyte accumulation in the nascent thickening was shown to inhibit SMC migration from the media into the intima, indicating that mononuclear leukocytes promote the formation of intimal thickening and possibly atherogenesis.

Materials and Methods

Antibodies

The mAb HP1/2 is a murine IgG_1 that binds to a functional epitope (characterized as epitope B) on the α_4 subunit (CD49d) of the human integrin VLA-4.[26] It cross-reacts with the respective rabbit homologue on mononuclear leukocytes but does not bind to rabbit neutrophils as evaluated by fluorescence-activated cell sorting.[27] HP1/2 was purified by protein A and gel filtration chromatography under endotoxin-free conditions[23,24] and used as a 6.5 mg/mL stock solution in sterile PBS.

Murine mAb 60.3 is of the IgG_{2a} subclass and recognizes the common β_2 chain of the membrane glycoprotein complex CD11/CD18 expressed on human leukocytes (B and T lymphocytes, monocytes, and the three subclasses of granulocytes but not red blood cells or ECs[28,29]). It has the advantage of cross-reactivity with the respective adhesion molecules on rabbit leukocytes.[27] mAb 60.3 was prepared according to Beatty et al,[28] and the concentration of the purified antibody protein in the stock solutions was 8.5 mg/mL in sterile saline.

In order to control the nonspecific effects of repeated treatment with foreign antibody proteins, we used the mouse anti-human antibody 17-1A produced by the hybridoma clone 1083-17-1A.[30,31] The hybrid clone secreted IgG_1 and IgG_{2a} at an early stage, but repeated cloning led to a loss of the myeloma γ-heavy chain. 17-1A binds with a high level of specificity to a surface antigen on human colorectal carcinoma. All mAb solutions contained <0.01 endotoxin unit per milligram.

Animals

Male New Zealand White rabbits obtained from Thomae GmbH (Biberach, FRG) were used for all experiments. They were fed standard rabbit pellets without lipid supplementation (Altromin GmbH) and were kept in the animal house for at least 1 week before the experiments were started. The animals entered the study at a body weight of 1.7 to 2.0 kg. All rabbits were exposed to the same method of transmural ES known to induce fibromuscular intimal thickening under normocholesterolemia,[15,16,18] but they were treated with the CD49d mAb HP1/2 (n=5), the combination of HP1/2 and the CD18 mAb 60.3 (n=5), or the nonspecific control antibody (n=3). For additional controls, eight rabbits received sterile saline instead of antibody.

Induction of Early Intimal Lesions and mAb Treatment

Early intimal lesions representing initial stages of fibromuscular thickening were induced in rabbit carotid arteries by applying direct-current impulses to the artery wall, as described previously.[17] Briefly, with the rabbits under anesthesia (15 mg metomidate hydrochloride and 0.1 mg IM fentanyl base per kilogram body weight), two graphite-coated gold electrodes (each measuring 1 mm × 5 mm) were attached to the adventitia of the right carotid artery and held in diametrical position by a flexible polytetrafluoroethylene (Teflon) cuff. The cuff was placed loosely around the outer surface of the arterial wall, so as not to interfere with the pulsation and compressibility of the vessel. The electrodes were connected via subcutaneous leads and a microplug fixed in the skull to an extracorporeal stimulation unit generating DC impulses (0.1 mA, 15 milliseconds, 10 Hz). These were applied to the artery wall of the freely moving rabbit in two sessions, starting 14 and 26 hours after surgery and lasting for 30 and 15 minutes, respectively. The left carotid served as an intraindividual control: Those rabbits treated with the combination of HP1/2 and mAb 60.3 or the nonspecific IgG received a so-called "silent" cuff where no current was applied; in case of the other treatments, the artery remained unmanipulated.

The protocol for administration of the different antibodies was identical to the one used in earlier experiments.[17] The antibodies were injected via the marginal ear vein three times at an interval of 12 hours, each at a dose of 2 mg/kg body wt. The first injection was given immediately before implantation of the electrodes; the second and third occurred 2 hours before the respective period of ES. This timing guaranteed that mAb administration interfered with the climax of leukocyte invasion into the arterial intima.[18] The saline control rabbits were subjected to the same schedule, but with corresponding injections of sterile saline. All antibody or saline treatments were performed with the animals under anesthesia (see above). Blood samples were drawn before each mAb or saline administration as well as at the end of the experiments for total and differential white blood cell counts. The experiments were terminated 36 hours after surgery in order to study the migration of SMCs as they began to cross the internal elastic lamina into the intimal compartment within the first 2 days of applying direct electrical current, as previously described.[16-18] The animals were anesthetized, and the carotids were perfused through the left ventricle with 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, at 80 to 100 mm Hg. After excision, the arteries were immersion-fixed in the same solution for at least 24 hours.

The experimental protocol was reviewed and approved by the animal care committee of the University of Tübingen.

Electron Microscopic Analysis

After we removed the cuff and ear-marked the anodal side of the artery wall where the eccentric intimal thickening develops,[15,16] the tissue was processed for transmission electron microscopy. Both the stimulated and the control carotid arteries were subdivided into rings ≈3 mm in length. All samples were postfixed in 1% buffered OsO_4 in 0.1 mol/L sodium cacodylate, dehydrated through graded alcohols, stained en bloc in alcoholic uranyl acetate, embedded in araldite, and transversely sectioned on an LKB ultramicrotome. Semithin sections were stained with toluidine blue. Ultrathin sections were taken from the anodal area of the block face as revealed by light electron microscopy, collected on 75-mesh copper grids, stained with lead citrate, and examined with a Zeiss EM 10.

The different cell types within the intimal cell population, ie, monocytes, lymphocytes, the subclasses of granulocytes, and SMCs, were identified on the basis of their ultrastructural features, as previously reported.[17] The subtypes of granulocytes, ie, neutrophils, basophils, and eosinophils, were clearly identifiable and distinguishable from monocytes and lymphocytes by their specific granules.[32] A sharp distinction between monocytes and lymphocytes was not always possible, depending on the cutting level. For quantification, they were therefore grouped as mononuclear leukocytes. However, the proportion of lymphocytes unambiguously identifiable by their fine structural characteristics (such as an oval nucleus, narrow rim of cytoplasm, tiny Golgi area, small number of lysosomes, and few large mitochondria) was determined, possibly underestimating their true quantities. Intimal SMCs were easily distinguished from leukocytes by the presence of a thin external lamina (sometimes only present in fragments), abundant micropinocytotic vesicles, myofilaments, and dense attachments on the cytoplasmic aspect of the membrane. Immunohistochemical analysis of paraformaldehyde-fixed paraffin-embedded tissue did not prove to be useful for cell typing in the early lesions under investigation, since the usual markers for identifying SMCs and macrophages could not be detected within the nascent thickening during the first 36 hours after electrode/cuff positioning. α-Actin, which is known to modulate its expression with the differentiation state of the cell,[33] was first detectable in intimal cells 8 days after the onset of ES, and the cell type–specific antigen of rabbit macrophages recognized by RAM-11 was expressed by subendothelial cells at the earliest 2 days after ES was started (data not shown).

For quantitative analysis, ultrathin cross sections through the midregion of the cuff-bearing artery segment were selected from a minimum of four different planes separated by at least 0.1 mm. The cellular composition of the entire cross-sectional area of the intimal lesion developing next to the anode and covered by ≈80 to 110 ECs was analyzed in a blind fashion. The numbers of intimal cells arranged in one or two cell layers beneath the endothelium were determined for each cell type and expressed in relation to the number of ECs overlying the lesion. The total count of ECs ranged from 454 to 677 per animal. The numbers of subendothelial cell profiles (with and without a sectioned part of the nucleus) belonging to a particular cell type were calculated per 100 ECs for each animal. The relative cellular composition was additionally determined by calculating the percentages of the different cell types within the intimal cell population.

Statistics

Data are presented as mean+SEM. Global effects of the treatment with specific antibodies on the composition of the intimal cell population were evaluated by one-way ANOVA after the data were normalized with logarithmic transformation. In the case of overall significance (attributed to $P<.05$), contrasts of selected pairs of group means were computed and adjusted by Holm-Bonferroni corrections.[34,35] The computer program SUPERANOVA was used in these computations.

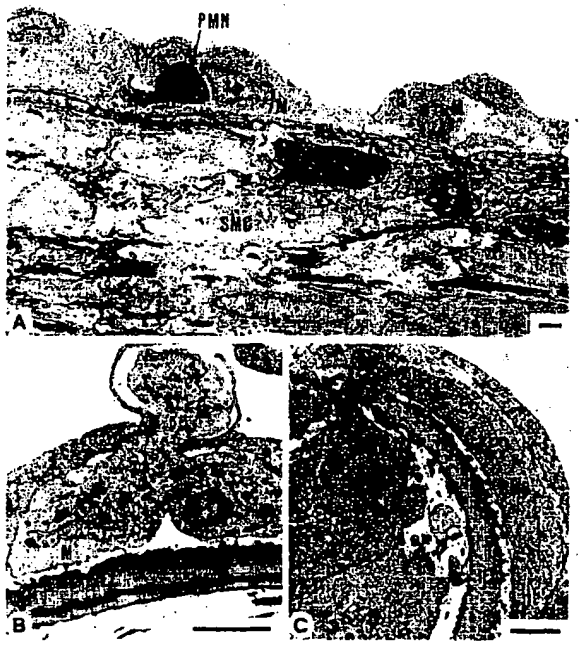

FIG 1. Electron micrographs showing characteristic features of intimal lesions induced by ES within 36 hours in carotid arteries from IgG-injected rabbits. A, Accumulation of PMNs and mononuclear leukocytes (M) between the intact endothelium (E) and the internal elastic lamina (IEL). B, Transendothelial migration of a monocyte (M). C, SMC in the process of migration from the media into the intima, as judged by its protrusion (arrowhead) through a pore of the IEL. Bars=2 μm.

To test for the effect of the antibody treatment on leukocyte counts in peripheral blood, the area under the curve (each curve representing leukocyte counts over time) was computed for each animal in the different treatment and control groups. The data were subjected to one-way ANOVA by using the logarithm of the area under the curve as the target variate.

Results

Early Intimal Lesions of Control Arteries

The nonmanipulated left carotid arteries of the control rabbits treated with saline had a normal appearance 36 hours after surgery. A morphologically intact endothelium covered the cell-free subendothelial space. No leukocytes were observed in association with the endothelial lining. In contrast, in the right carotid arteries exposed to two sessions of ES, leukocytes had populated the widened subendothelial space, forming lesions similar to those found in the stimulated carotid arteries of the rabbits injected with the nonspecific control antibody 17-1A. The leukocytes were arranged in one or two cell layers beneath a continuous endothelium with normal-appearing junctional complexes (Fig 1A). Additionally, they were seen adhering to, as well as trafficking through, the endothelium at this time (Fig 1B). When crossing the carotid endothelium, the leukocytes preferentially used the intercellular pathway through junctional complexes. PMNs and monocytes were the predominant cell types in the population of leukocytes invading the arterial intima, whereas lymphocytes, basophils, and eosinophils were only occasionally seen. The number of intimal PMNs and mononuclear leukocytes, including monocytes and lymphocytes, did not

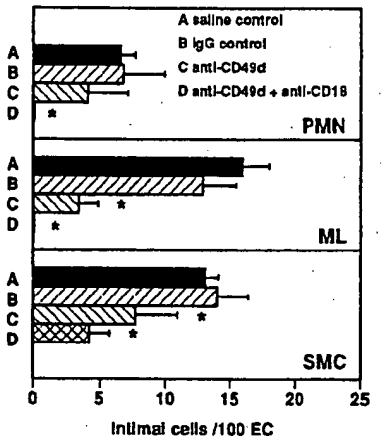

FIG 2. Bar graph showing accumulation of PMNs, mononuclear leukocytes (MLs), and SMCs within the intimal lesion induced by ES of rabbits treated with saline (n=8), nonspecific IgG (n=3), and anti-CD49d alone (n=5) or together with anti-CD18 (n=5). Antibodies and saline were injected intravenously at 0, 12, and 24 hours. The number of intimal cells is expressed in relation to 100 overlying ECs. For further experimental details, see "Materials and Methods." Data represent mean±SEM. *Significant difference from either control group treated with saline or nonspecific IgG ($P<.05$ by ANOVA and Holm-Bonferroni–adjusted contrasts).

significantly differ between the 17-1A–treated control and the saline-treated rabbits ($P>.35$, Fig 2). The proportion of unambiguously identified lymphocytes within the mononuclear leukocyte population was also similar in the two control groups and amounted to 8.3±3.5% in the 17-1A group and 7.9±2.5% in the saline group, respectively. Basophils and eosinophils were present within the stimulated intima in percentages <1%, without significant differences between the two control groups.

Not only did leukocytes accumulate in the stimulated intima of the control groups within 36 hours, but SMCs also started to migrate from the underlying media into the intimal compartment, which normally was void of SMCs. They were predominantly observed squeezing their way through pores in the internal elastic lamina and spreading out in the intima (Fig 1C). The number of SMCs that had already reached the intima was similar in the two control groups (17-1A control group, 14.2±2.5 per 100 ECs; saline-treated control group, 13.1±1.1 per 100 ECs; $P=.89$; Fig 2). SMC migration was the dominant factor contributing to the accumulation of SMCs in the intima at 36 hours, since proliferation of intimal SMCs was first detectable after 2 days of ES, as shown in former studies using bromdeoxyuridine labeling.[36]

In the left carotid arteries of the 17-1A control rabbits surrounded by silent cuffs, mononuclear leukocytes also migrated across the endothelium and populated the normally acellular intimal compartment, yet at a clearly reduced number compared with the electrically stimulated right carotid arteries (5.6±1.7 versus 12.6±2.5 per 100 ECs, $P=.017$). Likewise, SMCs were present in the intima under silent cuffs; their number was also significantly lower than in the corresponding segments exposed to ES (2.3±0.9 versus 14.2±2.5 per 100 ECs, $P=.04$).

Early Intimal Lesions After Treatment With Integrin-Recognizing mAbs

The systemic application of the anti–VLA-α_4 antibody HP1/2 did not affect PMN invasion into the electrically stimulated intima. PMNs were found to be adherent to, trafficking through, and lying beneath the endothelium. Their number within the nascent thickening was similar to that determined in the control arteries treated either with the nonspecific IgG ($P=.21$) or saline ($P=.08$), as indicated in Fig 2. Basophils and eosinophils, however, which constituted <1% of the intimal leukocyte population in each control group, were fully blocked in their invasion into the intima by HP1/2. Monocytes and lymphocytes still invaded the stimulated arterial intima after HP1/2 treatment, but their number was reduced to ≈30% of the IgG control value (Fig 2, $P=.0003$). Under these conditions, the number of intimal SMCs was significantly diminished by ≈50% compared with the control arteries (Fig 2; $P=.02$ versus saline, $P=.04$ versus IgG).

When anti–VLA-α_4 was given in combination with the anti-CD18 mAb 60.3, the invasion of monocytes, lym-

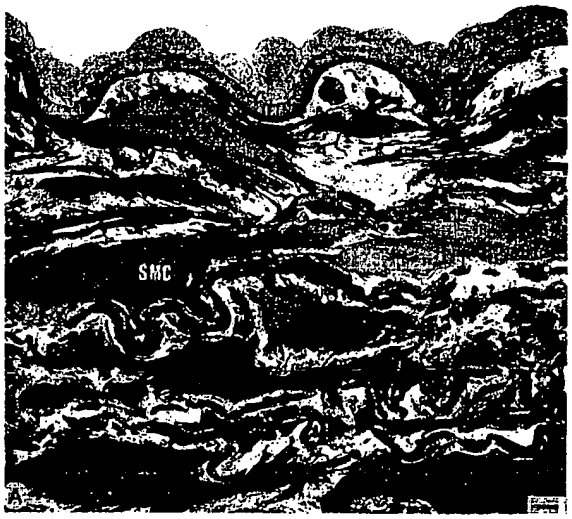

FIG 3. Electron micrographs showing characteristic features of intimal lesions induced by ES after treatment with anti-CD49d and anti-CD18. A, Representative part of a transverse section through the stimulated carotid artery 36 hours after electrode/cuff positioning and combined antibody treatment. The subendothelium is void of leukocytes. B, SMC migration into the intima despite complete abolishment of leukocyte invasion after administration of both antibodies. Migration occurs beneath interendothelial contacts (arrow) and in proximity to basal protrusions of ECs (arrowhead). IEL indicates internal elastic lamina; E, endothelium. Bars=2 μm.

phocytes, and granulocytes (all subsets) into the intima of the stimulated right carotid artery was completely abolished after 36 hours (Figs 2 and 3A). Now, the number of SMCs that had migrated into the intima in response to ES was reduced to a greater extent than after anti–VLA-α_4 treatment alone, reaching ≈30% of the IgG control value (Fig 2, P=.006). As shown in previous experiments performed under identical conditions with the anti-CD18 mAb alone,[17] total blockade of neutrophil invasion did not affect SMC migration. Thus, we can conclude that the reduction of mononuclear leukocyte infiltration by >70%, achieved in the present experiments, accounts for the inhibition of SMC movement from the media into the intimal compartment.

The number of intimal SMCs observed in the ES arteries after the combined antibody treatment (4.2±1.5 SMCs per 100 ECs) was similar to that found in the contralateral arteries surrounded by cuffs alone (3.7±1.5 SMCs per 100 ECs, P=.54). This minimal migratory response of SMCs was apparently unaffected by leukocytes, since in both the cuffed and ES arteries of the combined antibody treatment group leukocytes were absent. Interestingly, in those rabbits receiving IgG the cuffed but otherwise nonstimulated carotid arteries showed similar numbers of intimal SMCs (2.3±0.9 SMCs per 100 ECs, P=.41) as after combined antibody treatment.

Circulating Leukocytes

In the two control groups, total leukocyte counts remained on baseline levels within the first 24 hours after implantation of the electrodes without significant difference between the saline- and IgG-treated animals (Fig 4A, P=.12). In contrast, peripheral leukocyte counts of the other groups receiving either anti–VLA-α_4 alone or in combination with anti-CD18 increased dramatically within 24 hours (Fig 4A, overall significance at P=.0001 evaluated by one-way ANOVA). The combined use of anti–VLA-α_4 and anti-CD18 resulted in maximal leukocyte numbers of $51.7\pm5.7\times10^3/\mu L$, which was about eightfold the value found in the saline control arteries, whereas in the anti–VLA-α_4 group, leukocytes reached values of $16.1\pm1.7\times10^3/\mu L$.

Leukocytosis evoked by anti–VLA-α_4 was characterized by a marked increase in the number of lymphocytes within the experimental period from 0 to 24 hours (Fig 4B). PMN levels, however, remained within the range of the control levels during this time. When anti–VLA-α_4 and anti-CD18 were given together, both lymphocytes and PMN numbers increased, reaching significantly higher levels than those found in the animals treated with anti–VLA-α_4 alone (Fig 4B). Neutrophilia was based on a dramatic rise in juvenile PMNs from $1.0\pm0.03\times10^3$ at 0 hours to $10.6\pm0.9\times10^3/\mu L$ at 24 hours. Only in this group of combined antibody treatment did monocyte numbers clearly exceed control values at 24 hours (Fig 4B; P=.0002 versus IgG, P=.0009 versus saline).

Discussion

The central cellular feature of atherosclerotic lesions is the accumulation of SMCs, together with monocytes/macrophages and lymphocytes, in the arterial intima. The SMCs are either derived from existing intimal cells by proliferation or from cells that migrate in from the

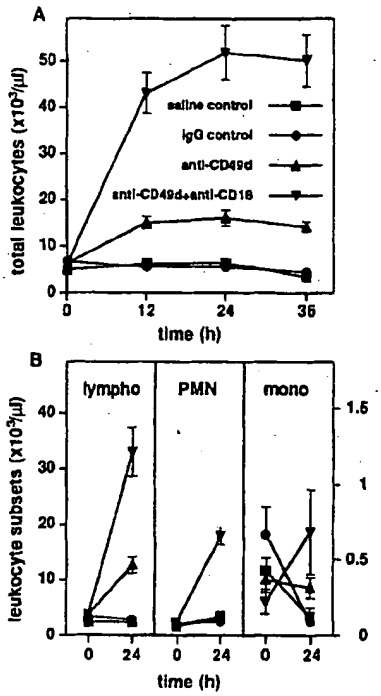

FIG 4. Line plot of number of circulating leukocytes in peripheral blood of rabbits treated with saline (n=8), nonspecific IgG (n=3), and anti-CD49d alone (n=5) or together with anti-CD18 (n=5) (antibody and saline administration similar to that described in Fig 2). A, Time dependence of total leukocyte counts after the different treatments. Data are expressed as mean±SEM. Leukocyte counts of the saline and IgG control group remained on baseline levels, whereas anti-CD49d alone and together with anti-CD18 increased leukocyte numbers (P=.0001 by ANOVA after determination of the area under the curve). B, Numbers of circulating lymphocytes (lympho), PMNs, and monocytes (mono) at 0 and 24 hours (note the different scale for monocyte counts).

underlying media. The present study provides insight into the mechanisms underlying the recruitment of monocytes and lymphocytes during experimental intimal thickening and the functional significance of these leukocytes for SMC migration from the media into the intima. The principal findings were that (1) monocytes and lymphocytes that initially invade the intimal thickening through the endothelium use pathways dependent on both the leukocytic integrins CD11/CD18 and VLA-4, and (2) the mononuclear leukocytes promote the directed movement of medial SMCs into the intima.

Contribution of CD18 and VLA-4 to Leukocyte Infiltration During Arterial Intimal Thickening

Using the model of electrically stimulated rabbit carotid artery, we recently demonstrated that the invasion of monocytes and lymphocytes into the arterial intima within the first 36 hours of the formation of fibromuscular thickening was in part CD18 dependent.[17] The present experiments extend this finding by showing that VLA-4 accounts for the CD18-independent portion of mononuclear leukocyte invasion during intimal thickening, since (1) the antibody HP1/2 recognizing the α_4 subunit of the β_1 integrin VLA-4 inhibited the stimulated influx of mononuclear leukocytes into the intima by ≈70% compared with the nonspecific IgG controls,

and (2) the combined use of the anti-VLA-α_4 and anti-CD18 mAb completely blocked mononuclear leukocyte emigration. Both monocytes and lymphocytes are known to use multiple pathways for adhering to ECs under both in vitro and in vivo conditions.[20,37-40] These include interactions between (1) the leukocytic receptors CD11a/CD18 or CD11b/CD18 and endothelial ICAM-1 and/or ICAM-2,[39-44] (2) VLA-4 and the inducible VCAM-1,[19,25,45,46] (3) the endothelial leukocyte adhesion molecule-1 (also designated E-selectin) and its leukocytic ligand(s),[37,47-49] and possibly (4) additional adhesion pathways that are as yet poorly characterized.[40,50] The dominance or hierarchy of the utilized pathways varies with the activation and differentiation state of the interacting cells[51] as well as with time.[27] In our experiments, the CD18-dependent and VLA-4–dependent interactions dominated and appeared to be sufficient for the recruitment of mononuclear leukocytes within the first 36 hours of experimental intimal thickening. Interestingly, despite blockade of both CD49d and CD18, some mononuclear leukocytes were observed adhering to the endothelium, suggesting a potential involvement of additional receptor/ligand pairs in the adhesion process. However, these adhesive interactions seemed to be "abortive" under the applied conditions, since they did not result in final accumulation in the subendothelial space of the carotid intima. The present study was not intended to distinguish whether the adhesion molecules are preferentially involved in adherence or transendothelial migration. As suggested from a substantial body of in vitro studies, the adhesion of monocytes,[52] lymphocytes,[53] and PMNs[52,54] to the endothelium is predominantly mediated by CD18-independent pathways, whereas the migration across the endothelium is dominated by CD18-dependent mechanisms. However, a significant VLA-4–dependent component in the chemotactic factor–induced migration of monocytes across activated endothelium has also been reported.[55] Future experiments are required to determine whether differences in the utilization of CD18 and VLA-4 during the adhesion and transendothelial migration also exist in our model.

The dependence of mononuclear leukocyte recruitment on VLA-4 and CD11/CD18 during fibromuscular thickening provides indirect evidence for the expression of the appropriate counterreceptors on the endothelial surface. VCAM-1 has been reported to be expressed by endothelial cells and by intimal subpopulations of SMCs and macrophages in both diet-induced[56-58] and human atherosclerotic lesions.[59,60] Additionally, increased immunoreactivity for ICAM-1 has been demonstrated at the arterial luminal surface of human atherosclerotic plaques.[59,61,62] On the basis of our findings, it may be speculated that both of these inducible endothelial adhesion molecules are expressed by the carotid endothelium during fibromuscular thickening in response to ES.

As far as granulocytes are concerned, the present work also provides information on the pathways underlying the immigration of basophils and eosinophils into the developing lesion. The occurrence of basophils and eosinophils in arterial thickening, although minimal compared with neutrophil invasion, is not a curiosity of our model. All subclasses of granulocytes have been described to be present in other models of experimental arteriosclerosis.[13,63-65] On their way into the electrically induced thickening, basophils and eosinophils used the VLA-4–dependent pathway, as judged by the complete abolishment of their influx by the anti-VLA-α_4 mAb. Neutrophil involvement, however, remained unaffected by this antibody. These results extend our previous observations that the anti-CD18 mAb 60.3 had no detectable effect on the accumulation of basophils and eosinophils in the electrically stimulated arterial intima.[17] Additionally, our data are in line with in vitro studies demonstrating that the adherence of basophils and eosinophils to cytokine-activated cultured ECs is dominated by the VLA-4 pathway and scarcely affected by CD18 antibodies,[21-23] whereas neutrophil binding was shown to be VLA-4 independent.[21,22,52]

Effect of Mononuclear Leukocytes on SMC Migration

The time frame in the present study (ie, termination of all experiments 36 hours after electrode implantation) was designed to examine the initial migration of SMCs as they start to move across the internal elastic lamina from the underlying media into the intimal compartment within the first 2 days of ES.[16-18] At this time, an increase in the replication rate of SMCs was not yet detectable in the intima,[36] suggesting that the SMCs identified at 36 hours within the ES intima were derived from cell migration but not cell proliferation.

From previous experiments, we can exclude PMNs as cellular mediators in SMC migration.[17] The present experiments indicate that the mononuclear leukocytes that accumulate within the nascent thickening promote SMC movement into the intima, since inhibition of mononuclear leukocyte infiltration by ≈70% achieved with anti-VLA-α_4 treatment resulted in reduced SMC migration. Moreover, the complete abolishment of mononuclear leukocyte influx by anti-VLA-α_4 plus anti-CD18 led to an even more pronounced reduction in the number of intimal SMCs. Monocytes/macrophages, which prevail in the population of infiltrating mononuclear leukocytes (at ≈90%), are good candidates for promoting SMC migration. They have the capacity to secrete not only proteolytic enzymes, which are capable of degrading the cage of extracellular matrix surrounding SMCs,[66-68] but also chemotactic substances for SMCs, eg, PDGF.[11] In fact, a rapid increase in PDGF-B chain expression was detected in rat carotid arteries exposed to the same method of ES as used in the present study[69]; however, without determining the cellular association. It is intriguing to raise the hypothesis that PDGF-B is indeed expressed in monocytes/macrophages in our model, as determined in human and nonhuman primate atherosclerosis[7] and that PDGF is one of the mediators of the migration-promoting effect on SMCs, as shown in other in vivo models of intimal thickening.[8,9,70] Whether the lymphocytes, which are also members of the mononuclear leukocyte population, also affect SMC migration, eg, by activating macrophages or by altering the responsiveness of SMCs to chemotactic factors, remains to be determined. It is less likely that the anti-CD18 or anti-VLA-α_4 antibody may exert a direct suppressive effect on SMC migration, since the CD18 integrins are exclusively expressed on leukocytes[20] and VLA-4 has so far not been demonstrated on adult vascular SMCs,[58,71] although α_4 expression has been detected on fetal SMCs.[72] However, it cannot be ex-

cluded that the antibodies indirectly affect SMC functions by interfering with SMC/leukocyte interactions mediated by ICAM-1 or VCAM-1, which both were shown to be expressed by SMCs in atherosclerotic lesions.[58,60-62] In the case of the combined treatment with anti–VLA-α_4 and anti-CD18, however, SMC/leukocyte interactions seem unlikely to occur, because leukocyte entry into the intima was completely blocked.

In addition to the leukocyte-dependent portion of SMC migration, our results also suggest that at least one third of the SMCs present in the early intimal lesion migrated independently of mononuclear leukocyte infiltration. This suggests that mononuclear leukocytes contribute only partially to the control of SMC migration and that additional systems are involved in regulating the migratory behavior of SMCs. ECs, for example, may be an additional regulatory component because of their ability to synthesize and release chemoattractants for SMCs[73,74] and to generate plasminogen activators that can induce and amplify the degradation of the matrix surrounding stimulated SMCs.[75] It is interesting to note that SMCs that migrated in the absence of mononuclear leukocytes into the intima were often observed closely interconnected with basal protrusions of ECs or beneath interendothelial contacts. It cannot be excluded that substances derived from the plasma that gain access to the arterial intima (eg, by enhanced endothelial permeability as observed after brief ES[16]) may also play a role in promoting SMC migration.

In conclusion, our findings show that in the rabbit model of electrically induced intimal thickening, the abolishment of mononuclear leukocyte invasion into the arterial intima reduced SMC migration, indicating a role for these leukocytes in stimulating lesion development and, possibly, in atherogenesis. The entry of mononuclear leukocytes was inhibited by blocking VLA-4–dependent and CD18-dependent adhesion mechanisms, suggesting that the expression of the respective endothelial counterreceptors is indirectly involved in mediating SMC migration.

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References

1. Munro JM, Cotran RS. The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Lab Invest.* 1988;58:249-261.
2. Hansson GK, Jonasson L, Seifert PS, Stemme S. Immune mechanisms in atherosclerosis. *Arteriosclerosis.* 1989;9:567-578.
3. Watanabe T, Tokunaga O, Fan J, Shimokama T. Atherosclerosis and macrophages. *Acta Pathol Jpn.* 1989;39:473-486.
4. Libby P, Hansson GK. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab Invest.* 1991;64:5-15.
5. Clinton SK, Libby P. Cytokines and growth factors in atherogenesis. *Arch Pathol Lab Med.* 1992;116:1292-1300.
6. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 1993;362:801-809.
7. Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden LT, Masuko H, Sato H. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science.* 1990;248:1009-1012.
8. Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science.* 1991;253:1129-1132.
9. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest.* 1992;89:507-511.
10. Shimokado K, Raines EW, Madtes DK, Barret TB, Benditt EP, Ross R. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell.* 1985;43:277-286.
11. Martinet Y, Bitterman PB, Mornex JF, Grotendorst GR, Martin GR, Crystal RG. Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF-like activity. *Nature.* 1986;319:158-160.
12. Hansson GK, Holm J, Fotev Z, Hedrich H-J, Fingerle J. T lymphocytes inhibit the vascular response to injury. *Proc Natl Acad Sci U S A.* 1991;88:10530-10534.
13. Prescott MF, Karboski McBride C, Court M. Development of intimal lesions after leukocyte migration into the vascular wall. *Am J Pathol.* 1989;135:835-846.
14. Hancock WW, Adams DH, Wyner LR, Sayegh MH, Karnovsky MJ. CD4$^+$ mononuclear cells induce cytokine expression, vascular smooth muscle cell proliferation, and arterial occlusion after endothelial injury. *Am J Pathol.* 1994;145:1008-1014.
15. Betz E, Schlote W. Responses of vessel walls to chronically applied electrical stimuli. *Basic Res Cardiol.* 1979;74:10-20.
16. Kling D, Holzschuh T, Betz E. Temporal sequence of morphological alterations in artery walls during experimental atherogenesis: occurrence of leukocytes. *Res Exp Med (Berl).* 1987;187:237-250.
17. Kling D, Fingerle J, Harlan JM. Inhibition of leukocyte extravasation with a monoclonal antibody to CD18 during formation of experimental intimal thickening in rabbit carotid arteries. *Arterioscler Thromb.* 1992;12:997-1007.
18. Kling D, Holzschuh T, Betz E. Recruitment and dynamics of leukocytes in the formation of arterial intimal thickenings: a comparative study with normo- and hypercholesterolemic rabbits. *Atherosclerosis.* 1993;101:79-96.
19. Hemler ME, Elices MJ, Parker C, Takada Y. Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. *Immunol Rev.* 1990;114:45-65.
20. Carlos TM, Harlan JM. Membrane proteins involved in phagocyte adherence to endothelium. *Immunol Rev.* 1990;114:5-28.
21. Bochner BS, Luscinskas FW, Gimbrone MA, Newman W, Sterbinsky SA, Derse-Anthony CP, Klunk D, Schleimer RP. Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. *J Exp Med.* 1991;173:1553-1557.
22. Walsh GM, Mermod JJ, Hartnell A, Kay AB, Wardlaw AJ. Human eosinophil, but not neutrophil, adherence to IL-1-stimulated human umbilical vascular endothelial cells is $\alpha_4\beta_1$ (very late antigen-4) dependent. *J Immunol.* 1991;146:3419-3423.
23. Weg VB, Williams TJ, Lobb RR, Nourshargh S. A monoclonal antibody recognizing very late activation antigen-4 inhibits eosinophil accumulation in vivo. *J Exp Med.* 1993;177:561-566.
24. Podolsky DK, Lobb R, King N, Benjamin CD, Pepinsky B, Sehgal P, deBeaumont M. Attenuation of colitis in the cotton-top tamarin by anti-α_4 integrin monoclonal antibody. *J Clin Invest.* 1993;92:372-380.
25. Elices MJ, Osborn L, Takada Y, Crouse C, Lubowskyj S, Hemler ME, Lobb RR. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* 1990;60:577-584.
26. Pulido R, Elices MJ, Campanero MR, Osborn L, Schiffer S, Garcia-Pardo A, Lobb R, Hemler ME, Sanchez-Madrid F. Functional evidence for three distinct and independently inhibitable adhesion activities mediated by the human integrin VLA-4. *J Biol Chem.* 1991;266:10241-10245.
27. Winn RK, Harlan JM. CD18-independent neutrophil and mononuclear leukocyte emigration into the peritoneum of rabbits. *J Clin Invest.* 1993;92:1168-1173.
28. Beatty PG, Ledbetter JA, Martin PJ, Price TH, Hansen JA. Definition of a common leukocyte cell-surface antigen (Lp95-150) associated with diverse cell-mediated immune functions. *J Immunol.* 1983;131:2913-2918.
29. Harlan JM, Killen PD, Senecal FM, Schwartz BR, Yee EK, Taylor RF, Beatty PG, Price TH, Ochs HD. The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro. *Blood.* 1985;66:167-178.
30. Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somat Cell Mol Genet.* 1979;5:957-972.

31. Herlyn M, Steplewski Z, Herlyn D, Koprowski H. Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. *Proc Natl Acad Sci U S A.* 1979;76:1438-1442.
32. Wetzel BK, Horn RG, Spicer SS. Fine structural studies on the development of heterophil, eosinophil, and basophil granulocytes in rabbits. *Lab Invest.* 1967;16:349-380.
33. Kocher O, Gabbiani G, Reidy MA, Cokay MS, Peters H, Huttner I. Phenotypic features of smooth muscle cells during the evolution of experimental carotid artery intimal thickening: biochemical and morphologic studies. *Lab Invest.* 1991;65:459-470.
34. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Stat.* 1979;6:65-70.
35. Bauer P. Multiple testing in clinical trials. *Stat Med.* 1991;10: 871-890.
36. Betz E, Strohschneider T. The time course in the development of experimentally induced atheromas. In: Crepaldi G, Gotto AM, Manzato E, Baggio G, eds. *Atherosclerosis VIII.* Amsterdam, Netherlands/New York, NY/Oxford, UK: Excerpta Medica; 1989: 141-144.
37. Springer TA. Adhesion receptors of the immune system. *Nature.* 1990;346:425-434.
38. Jutila MA. Leukocyte traffic to sites of inflammation. *APMIS.* 1992; 100:191-201.
39. Faruqi RM, DiCorleto PE. Mechanism of monocyte recruitment and accumulation. *Br Heart J.* 1993;69(suppl):S19-S29.
40. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood.* 1994;84:2068-2101.
41. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol.* 1988; 107:321-331.
42. Rothlein R, Dustin ML, Marlin SD, Springer TA. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J Immunol.* 1986;137:1270-1274.
43. Makgoba MW, Sanders ME, Luce GEG, Dustin ML, Springer TA, Clark EA, Mannoni P, Shaw S. ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature.* 1988;331: 86-88.
44. Staunton DE, Dustin ML, Springer TA. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature.* 1989;339:61-64.
45. Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosso M, Osborn L, Newman B, Lobb RR, Harlan JM. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine activated cultured endothelial cells. *Blood.* 1990;76:965-970.
46. Rice GE, Munro JM, Bevilacqua MP. Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes: a CD11/CD18-independent adhesion mechanism. *J Exp Med.* 1990;171:1369-1374.
47. Graber N, Gopal TV, Wilson D, Beall LD, Polte T, Newman W. T-cells bind to cytokine-activated endothelial cells via a novel, inducible sialoglycoprotein and ELAM-1. *J Immunol.* 1990;145: 819-830.
48. Shimizu Y, Shaw S, Graber N, Gopal TV, Horgan KJ, van Seventer GA, Newman W. Activation independent binding of human memory T cells to adhesion molecule ELAM-1. *Nature.* 1991;349: 799-802.
49. Lenter M, Levinovitz A, Isenmann S, Vestweber D. Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. *J Cell Biol.* 1994;125:471-481.
50. Meerschaert J, Furie MB. The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. *J Immunol.* 1995;154:4099-4112.
51. Shimizu Y, Newman W, Gopal TV, Horgan KJ, Graber N, Beall LD, van Seventer GA, Shaw S. Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J Cell Biol.* 1991;113:1203-1212.
52. Hakkert BC, Kuijpers TW, Leeuwenberg JFM, van Mourik JA, Roos D. Neutrophil and monocyte adherence to and migration across monolayers of cytokine-activated endothelial cells: the contribution of CD18, ELAM-1, and VLA-4. *Blood.* 1991;78: 2721-2726.
53. Kavanaugh AF, Lightfoot E, Lipsky PE, Oppenheimer-Marks N. Role of CD11/CD18 in adhesion and transendothelial migration of T cells: analysis utilizing CD18-deficient T cell clones. *J Immunol.* 1991;146:4149-4156.
54. Smith CW, Rothlein R, Hughes BJ, Marsicalco MM, Schmalstieg FC, Rudloff HE, Anderson DC. Recognition of an endothelial determinant for CD18-dependent human neutrophil adherence and transendothelial migration. *J Clin Invest.* 1988;82:1746-1756.
55. Chuluyan HE, Issekutz AC. VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. *J Clin Invest.* 1993;92:2768-2777.
56. Cybulsky MI, Gimbrone MA. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science.* 1991;261:788-791.
57. Li H, Cybulsky MI, Gimbrone MA, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine regulatable mononuclear leukocyte adhesion molecule, in rabbit endothelium. *Arterioscler Thromb.* 1992;13:197-204.
58. Li H, Cybulsky MI, Gimbrone MA, Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. *Am J Pathol.* 1993;143: 1551-1559.
59. Davies MJ, Gordon JL, Gearing AJH, Pigott R, Woolf N, Katz D, Kyriakopoulos A. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J Pathol.* 1993;171:223-229.
60. O'Brien KD, Allen MD, McDonald TO, Chait A, Harlan JM, Fishbein D, McCarty J, Ferguson M, Hudkins K, Benjamin CD, Lobb R, Alpers CE. Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques: implications for the mode of progression of advanced coronary atherosclerosis. *J Clin Invest.* 1993;92:945-951.
61. Printseva OY, Peclo MM, Gown AM. Various cell types in human atherosclerotic lesions express ICAM-1: further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3. *Am J Pathol.* 1992;140:889-896.
62. Poston RN, Haskard DO, Coucher JR, Gall NP, Johnson-Tidey RR. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. *Am J Pathol.* 1992;140:665-673.
63. Trillo AA. The cell population of aortic fatty streaks in African green monkey with special reference to granulocytic cells: an ultrastructural study. *Atherosclerosis.* 1982;43:253-275.
64. Stary HC, Malinow MR. Ultrastructure of experimental coronary artery atherosclerosis in cynomolgus macaques. *Atherosclerosis.* 1982;43:151-175.
65. Faggiotto A, Ross R. Studies of hypercholesterolemia in the nonhuman primate, II: fatty streak conversion to fibrous plaque. *Arteriosclerosis.* 1984;4:341-356.
66. Nathan CF, Murray HW, Cohn ZA. The macrophage as an effector cell. *N Engl J Med.* 1980;303:622-626.
67. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Suenram CA, Graves DT, Rozek MM, Edwards EH, Delgado R. Monocyte-macrophage participation in atherogenesis: inflammatory components of pathogenesis. *Semin Thromb Hemost.* 1986;12:79-86.
68. Campbell JH, Campbell GR. The macrophage as an initiator of atherosclerosis. *Clin Exp Pharmacol Physiol.* 1990;18:81-84.
69. Bilder GE, Kasiewski CJ, Costello RJ, Hodge TG, Perrone MH. Electrode cuff-induced changes in DNA and PDGF gene expression in the rat carotid artery. *Atherosclerosis.* 1993;100: 103-112.
70. Jackson CL, Raines EW, Ross R, Reidy MA. Role of endogenous platelet-derived growth factor in arterial smooth muscle cell migration after balloon catheter injury. *Arterioscler Thromb.* 1993; 13:1218-1226.
71. Mechtersheimer G, Barth T, Quentmeier A, Möller P. Differential expression of β_1 integrins in nonneoplastic smooth and striated muscle cells and in tumors derived from these cells. *Am J Pathol.* 1994;144:1172-1182.
72. Sheppard AM, Onken MD, Rosen GD, Noakes PG, Dean DC. Expanding roles for α_4 integrin and its ligands in development. *Cell Adhes Commun.* 1994;2:27-43.
73. DiCorleto PE, Chisolm GM. Participation of the endothelium in the development of the atherosclerotic plaque. *Prog Lipid Res.* 1986;25:365-374.
74. Zerwes HG, Risau W. Polarized secretion of a platelet-derived growth factor-like chemotactic factor by endothelial cells in vitro. *J Cell Biol.* 1987;105:2037-2041.
75. Levin EG, Loskutoff DJ. Cultured bovine endothelial cells produce both urokinase and tissue-type plasminogen activators. *J Cell Biol.* 1982;94:631-636.

Inhibition of Leukocyte Extravasation With a Monoclonal Antibody to CD18 During Formation of Experimental Intimal Thickening in Rabbit Carotid Arteries

Dorothee Kling, Jürgen Fingerle, and John M. Harlan

In the rabbit model of electrically induced intimal thickening, the adherence processes of different leukocyte subsets as well as the functional significance of leukocyte invasion in the initial migration of smooth muscle cells (SMCs) into the intima were studied by using monoclonal antibody (MAb) 60.3 (directed to the leukocyte adherence glycoprotein CD18), a known potent inhibitor of leukocyte adhesive functions. In control carotid arteries exposed to two periods of electrical stimulation within 36 hours, leukocytes, including all granulocyte subsets, monocytes, and lymphocytes, invaded the cell-free subendothelium. Concomitantly, SMCs were observed to migrate from the media into the intima. In the MAb 60.3–treated rabbits, however, neutrophil emigration into the stimulated arteries was abolished, whereas mononuclear leukocyte accumulation in the intima was only partially inhibited, indicating a complete CD18-dependent mechanism for neutrophil extravasation and additional receptor–ligand systems for the emigration of mononuclear leukocytes. SMCs moved into the intima despite complete blockage of neutrophils and the reduced accumulation of mononuclear cells within the subendothelium after MAb administration. These results preclude neutrophils as initiators of SMC migration into the intima. The influence of mononuclear cells on the migratory behavior of SMCs in intimal thickening formation, however, needs further elucidation. (*Arteriosclerosis and Thrombosis* 1992;12:997–1007)

KEY WORDS • **intimal thickening** • **endothelium** • **leukocyte extravasation** • **leukocyte adhesion molecules** • **CD18 complex** • **smooth muscle cell migration** • **rabbit carotid artery**

Adherence of monocytes and lymphocytes to the arterial endothelium, their transendothelial migration, and their accumulation within the intima have been frequently described as early events in human and experimental atherosclerosis.[1–14] In advanced lesions also, significant amounts of mononuclear cells have been identified.[6,15–20] Evidence exists that the mononuclear leukocytes, in addition to the well-recognized lipid-scavenging function of monocytes/macrophages, may contribute to lesion initiation and progression by secreting a variety of inflammatory mediators, cytokines, and growth factors (reviewed in References 21–24). Granulocytic cells, although attracting minor attention, have also been reported to occur in both human atherosclerotic plaques and experimentally induced intimal thickenings.[6,13,16,17,25] Their functional significance in the development of intimal lesions is still a matter of speculation.[25–27]

In the model that induces intimal thickening in rabbit carotid arteries in response to electrical stimulation (ES),[28] we previously demonstrated a massive initial invasion of both granulocytes and mononuclear cells into the developing lesion. This event was observed in the presence of a continuous yet functionally and structurally altered endothelium, and it preceded the accumulation of smooth muscle cells (SMCs) within the arterial intima.[29,30] Within 4 weeks, intimal thickenings, primarily consisting of SMCs and to a minor extent mononuclear cells, developed in normocholesterolemic rabbits.[29] These thickenings resembled fibromuscular intimal lesions formed either after balloon catheter injury[31] or by perivascular manipulation.[13,32–34] Initial events like endothelial damage and leukocyte involvement, however, differed between the various models. In addition, a cholesterol-rich diet fed during ES resulted in atheroma-type lesions containing macrophage-derived foam cells as well as lipid-laden SMCs and showing signs of necrosis,[28,35] hence bearing some of the features of human atherosclerosis.

In the present study, interest was focused on 1) the processes mediating leukocyte adhesion and emigration into the vessel wall in this animal model and 2) the functional relation that might exist between leukocyte invasion and SMC migration into the arterial intima. Our tool in elucidating these problems was the monoclonal antibody (MAb) 60.3, which recognizes the leukocyte membrane glycoprotein CD18 and which has proven to be a potent inhibitor of leukocyte adherence

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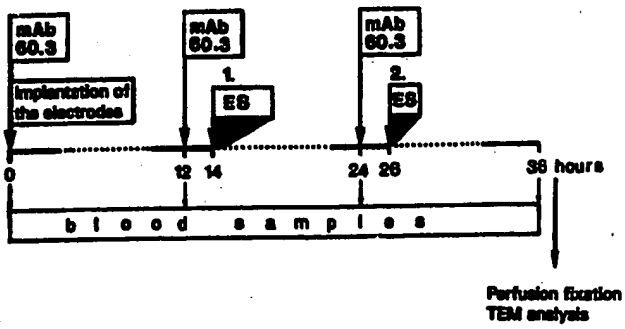

FIGURE 1. *Experimental design for electrical stimulation (ES) of carotid arteries from rabbits treated with monoclonal antibody (MAb) 60.3. Control rabbits were exposed to the same schedule but received injections of saline rather than MAb 60.3. TEM, transmission electron microscopy.*

to the endothelium in different models (reviewed in Reference 36). At the level of the electron microscope, we examined whether MAb 60.3 was able to interfere with adhesion and diapedesis of leukocytes in vivo after endothelial activation by weak ES. Furthermore, we tested whether inhibited leukocyte invasion would modulate the migration of medial SMCs into the intima. The model of electrically induced intimal thickenings is particularly suited to these investigations, as both leukocyte invasion and signs of SMC migration are prominent features within the first 2 days of the stimulation program, hence avoiding prolonged antibody treatment.

Methods

Monoclonal Antibody

MAb 60.3 is a murine immunoglobulin (Ig) G_{2a} antibody that recognizes all three heterodimers of the membrane glycoprotein complex CD11/CD18 expressed on human leukocytes (B and T lymphocytes, monocytes, and polymorphonuclear leukocytes but not red blood cells, platelets, or cultured endothelial cells[37]). It binds to a functional epitope on the common β_2-chain (CD18) and has the advantage of cross-reactivity with cell-adhesion molecules of leukocytes from other species (reviewed in Reference 38). MAb 60.3 was prepared according to Beatty et al.[39] Briefly, a BALB/c mouse was immunized with alloantigen-activated T cells. Ascites fluids were obtained from pristine primed BALB/c mice inoculated intraperitoneally with 5×10^6 hybridoma cells. The antibody was purified by solid-phase absorption on staphylococcal protein A columns. The antibody concentrations in the stock solutions were 2–5 mg/ml. In cases where 0.01% NaN_3 was added to the antibody solutions, it was removed by exhaustive dialysis against sterile phosphate-buffered saline.

Animals and Experimental Design

Male New Zealand White rabbits (1.4–1.8 kg body weight, $n=12$), obtained from Thomae GmbH (Biberach, FRG), were used for the study. They were fed a normal rabbit standard diet (Altromin GmbH, Lage, FRG) during acclimatization in the animal department for at least 1 week as well as during the experiment. The right carotid arteries of the rabbits were exposed to transmural ES, which is known to elicit intimal thickening.[28] In addition, one group of animals ($n=7$) received MAb 60.3 before as well as during ES; the others ($n=5$) received sterile saline as a control. The following experimental protocol was used. After anesthesia of the rabbits (15 mg metomidate hydrochloride and 0.1 mg fentanyl base/kg body wt i.m.), two graphite-coated gold electrodes were attached to the adventitia of the right carotid artery and held in a diametrical position by a flexible Teflon cuff. The electrodes were subcutaneously connected with a plastic socket in the skull, as already described.[28,29] With this stimulation device, direct-current impulses (0.1 mA, 15 msec/impulse, 10 Hz) were applied to the artery wall of the conscious rabbit in two sessions; the first occurred 14 hours after surgery and lasted for 30 minutes, and the second 12 hours later for a duration of 15 minutes (Figure 1). The left carotid artery remained electrically unstimulated and served as an intraindividual control. Simultaneously with the implantation of the electrodes, the first injections of MAb 60.3 or saline were given via the marginal ear vein. Five animals were treated with 2 mg MAb 60.3/kg body wt, a dose that had been determined to completely saturate CD18 binding sites on polymorphonuclear neutrophils (PMNs),[40] and two animals received 3 mg MAb 60.3/kg body wt. The control rabbits ($n=5$) were injected with 2 ml saline, a volume equivalent to that given the MAb-treated animals. Under anesthesia (8 mg metomidate hydrochloride and 0.07 mg fentanyl base/kg body wt i.m.), the MAb or saline injections were repeated twice at intervals of 12 hours, i.e., the second and third occurred 2 hours before the respective period of ES (Figure 1). Blood samples were drawn immediately before each MAb or saline administration, as well as at the end of the experiments for determination of total and differential leukocyte counts. Thirty-six hours after surgery, animals were anesthetized and their carotid arteries perfused through the left ventricle at a pressure of 80–100 mm Hg with 0.1 M cacodylate-buffered 1.25% glutaraldehyde, as already described.[29] The carotid arteries were carefully excised, the cuff was removed after earmarking the adventitial side next to the anode, and the vessels were stored in fixative (same composition as above).

The experimental protocol was reviewed and approved by the University of Tübingen Animal Care Committee.

Tissue Processing for Electron Microscopy

After immersion-fixation for at least 24 hours, the arteries were processed for transmission electron microscopy. The cuff-bearing artery segments were bisected, allowing examination of the vessel wall changes in the mid-region of the cylindrical segment. The left carotid arteries were also subdivided into rings approximately 3 mm long. All samples were postfixed in 1% OsO_4, dehydrated through graded alcohols, stained en bloc in alcoholic uranyl acetate, embedded in Araldite,

FIGURE 2. *Photomicrographs of leukocytes associated with the endothelium of electrically stimulated carotid arteries from rabbits not treated with MAb 60.3.* Panel A: *Polymorphonuclear neutrophil (PMN) sending several protrusions into interendothelial gaps. Note the close membrane apposition between the neutrophil and the endothelial cells (E).* Panel B: *Monocyte (M) protruding through the endothelium (E) to the subendothelial space (SES). IEL, internal elastic lamina.* Panel C: *Lymphocyte (L) characterized by an oval nucleus with deep indentations and a narrow rim of clear cytoplasm is attached to the endothelium (E).* Panel D: *Eosinophilic granulocyte (EO) containing crystalloid structures (arrows) within its granules is lying between two endothelial cells (E). Bars=1 μm.*

In the MAb-treated rabbits, the accumulation of leukocytes in the intima next to the anode of carotid arteries exposed to two periods of ES was clearly reduced. As shown in Figure 4, PMNs were totally absent in the intima of MAb-receiving animals, whereas basophil and eosinophil counts remained unchanged.

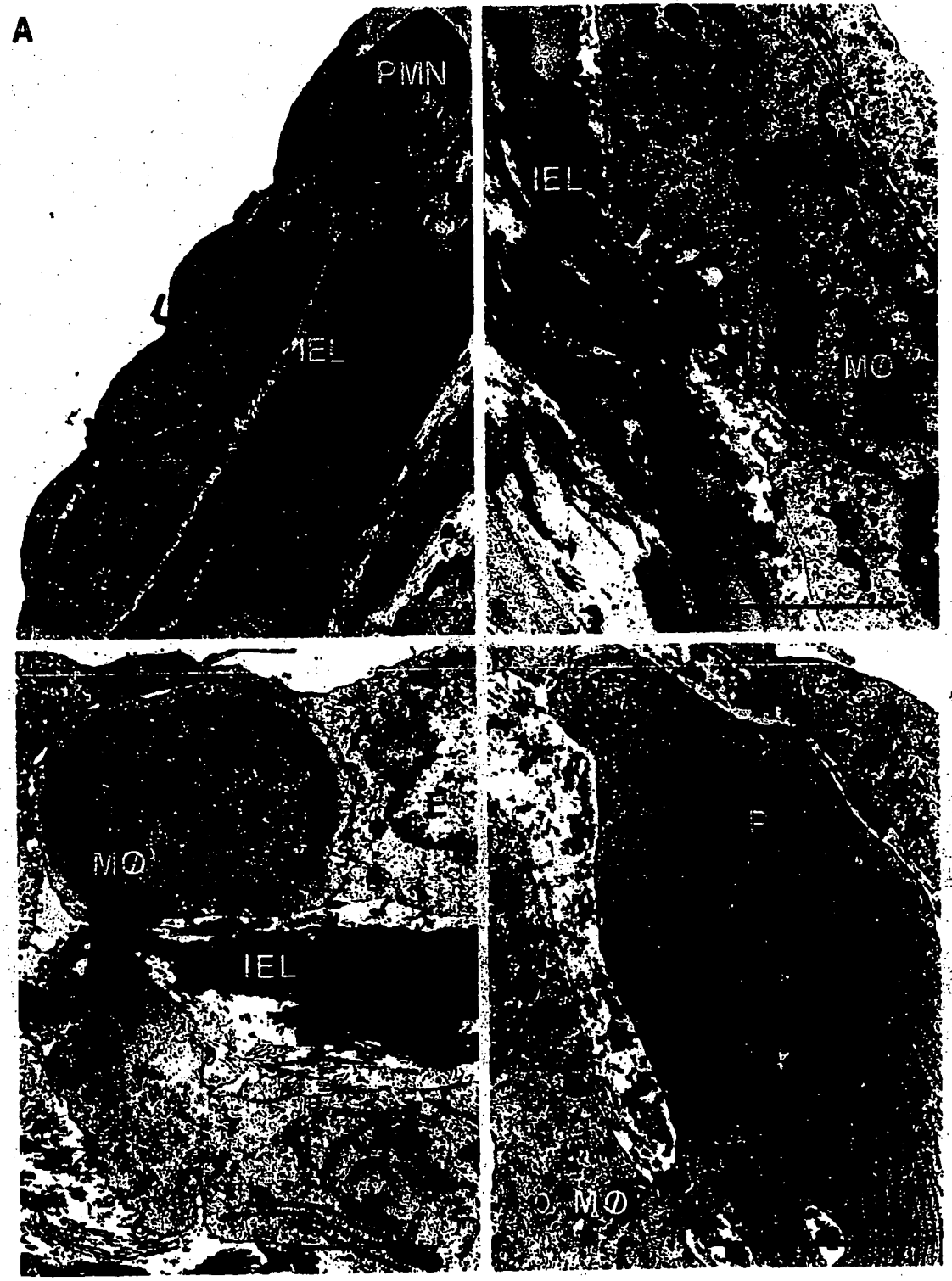

FIGURE 3. *Photomicrographs of leukocytes accumulating within the subendothelium from control rabbit carotid arteries after exposure to the stimulation program for 36 hours.* **Panel A:** *Polymorphonuclear neutrophil (PMN) and monocytes/macrophages (MØ) are arranged in a single layer between the intact endothelium (E) and the internal elastic lamina (IEL).* **Panel B:** *Villous cytoplasmic processes (arrows) of a macrophage are protruding toward the internal elastic lamina. Note fragmentation of the lamina and a pseudopod of a smooth muscle cell (arrowhead) next to the protrusions.* **Panel C:** *Macrophage squeezing through the internal elastic lamina. E, endothelium.* **Panel D:** *Plasma cell (P) with its distinctive well-packed, rough endoplasmic reticulum lying beneath the endothelium and making contact with a macrophage. Bars=2 μm.*

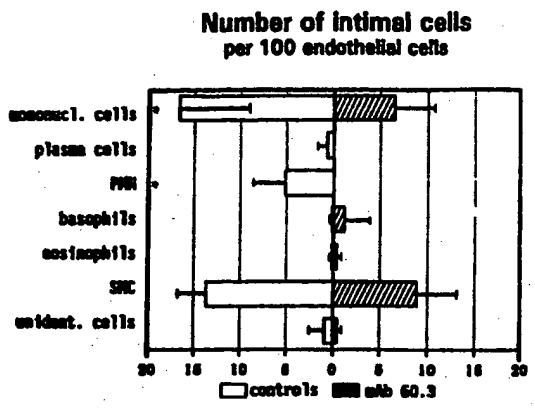

FIGURE 4. *Bar graph showing accumulation of different cell types within the rabbit carotid intima after exposure to electrical stimulation for 36 hours and treatment of the animals with either monoclonal antibody (MAb) 60.3 (2 mg/kg) or saline (control). Number of intimal cells belonging to a particular cell type is expressed in relation to 100 overlying endothelial cells. Data given represent mean+SD of five animals in each experimental group. Asterisks indicate a significant difference at* $p<0.05$ *compared with saline-injected control rabbits (Wilcoxon test). Mononucl., mononuclear; PMN, polymorphonuclear neutrophils; SMC, smooth muscle cells; unident., unidentified.*

Degenerating granulocytes were rarely seen attached to the endothelium, but they were never found within the subendothelium. The invasion of both monocytes and lymphocytes into the arterial intima under ES conditions was not completely prevented by applying the antibody in doses of 2 mg/kg body wt. Mononuclear cells were observed either migrating through or already lying beneath an intact endothelial lining. However, the number of the subendothelially located mononuclear cells was significantly lower than that in the control rabbits and was approximately one third of the value established in the saline-injected animals (Figure 4; one-sided Wilcoxon test, $p<0.05$). Even increasing the antibody dose to 3 mg/kg body wt was not effective in abolishing emigration of monocytes and lymphocytes. In two rabbits a dose of 3 mg/kg body wt resulted in 7±2 intimal mononuclear cells, which was in the same range as in the animals injected with 2 mg/kg (i.e., 6±4 mononuclear cells/100 ECs). Plasma cells were not seen within the intima of the stimulated arteries of the MAb-treated animals.

Smooth Muscle Cell Migration Into the Intima

Responses to two sessions of ES were characterized not only by the invasion of leukocytes into the subendothelium but also by SMC migration from the media into the intima. Thus, in the carotid arteries of control animals exposed to the described stimulation schedule, after 36 hours SMCs were frequently seen in the process of sending pseudopods through pores of the internal elastic lamina and spreading out into the intima (Figure 5). These SMCs exhibited ultrastructural features of the intermediate phenotype (i.e., clearly visible myofilaments in the peripheral zone, an enlarged perinuclear space free of myofilaments but enriched with mitochondria, and rough endoplasmic reticulum). In some instances, the nucleated part of the SMC had already reached the intima where leukocytes had predominated thus far. The proportion of muscle cells within the entire subendothelial cell population amounted to 39±10%.

In the MAb-treated rabbits SMC movement into the intima was not prevented despite reduced accumulation of leukocytes (Figure 4). It is important to note that migrating SMCs were often found in areas where monocytes/macrophages had accumulated in the intima.

Total and Differential Leukocyte Counts in Peripheral Blood

Total leukocyte counts of ES rabbits treated with 2 mg MAb 60.3/kg body wt were compared with those of animals injected with saline (Figure 6). In the control animals a moderate increase in the number of peripheral white blood cells was established 12 hours after implantation of the electrodes and the first saline injection, indicating an inflammatory reaction to the surgical procedure of electrode placement. Leukocyte counts, however, returned to baseline levels at 36 hours ($3.0\pm1.6\times10^3/\mu l$). In animals given MAb 60.3 there was marked leukocytosis at all times studied (maximally, $19.8\pm2.3\times10^3/\mu l$ at 24 hours), showing a significantly different course from animals not treated with MAb (quadratic analysis of covariance, $p<0.05$).

With regard to the different leukocyte subsets (Figure 7), a cell type–specific effect of both MAb treatment and time was determined by using multiway analysis of variance ($p<0.01$). In particular, the relative amounts of PMNs (i.e., juvenile and mature forms together) increased within the first third of the examination interval in both experimental groups. However, in the MAb-treated animals the rise in the percentages of the juvenile PMNs was more pronounced (Figure 7). They reached a level of 20±5% at 12 hours, which is significantly different from the respective value in the control group (6±3%, $p<0.05$). This difference in the amount of juvenile PMNs between MAb-treated and control animals was maintained over the entire period studied. Both total and differential leukocyte counts revealed that MAb administration resulted in marked neutrophilia, which was at least partly based on an increased mobilization of juvenile PMNs.

Discussion

Using the model of electrically induced intimal thickening in the rabbit carotid artery, we have demonstrated that 1) MAb 60.3 completely abolished the initial emigration of PMNs as well as their accumulation within the intima, 2) MAb 60.3 only partially inhibited the invasion of mononuclear cells, and 3) the reduced infiltration of leukocytes did not prevent SMC migration into the intima.

The abolishment of PMN accumulation in the ES intima by MAb 60.3 suggests that PMN adherence and/or extravasation are primarily CD18-mediated processes. This finding confirms in vitro data, which showed that MAb 60.3 inhibited adhesion of stimulated human PMNs to human EC monolayers,[37,44] and it is in accordance with a number of results from in vivo studies in which the antibody profoundly hindered neutrophil emigration in response to inflammatory stimuli (reviewed in Reference 36). Our data were obtained by a

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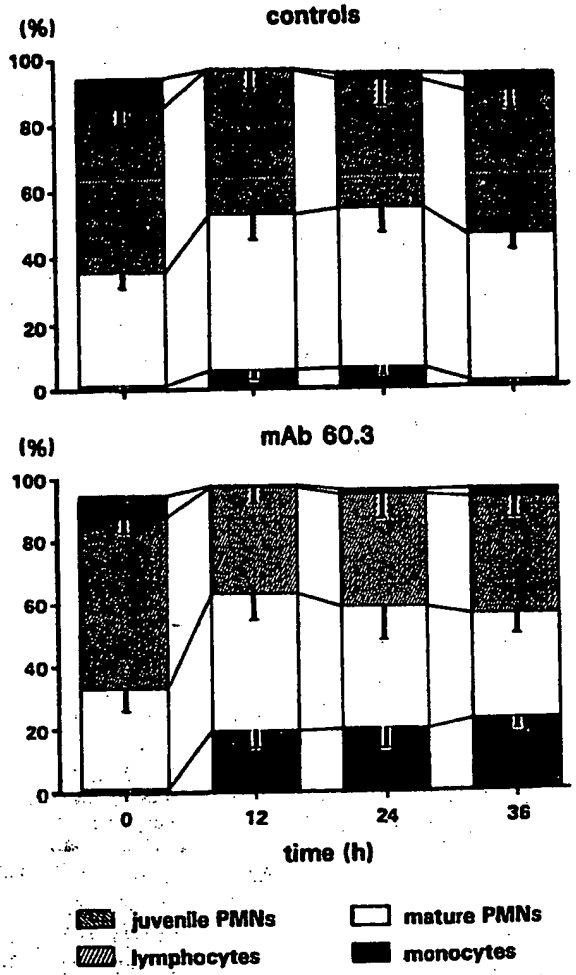

FIGURE 7: *Bar graphs showing percentages of different leukocyte subsets in peripheral blood before and after injection of saline (controls;* upper panel*) and monoclonal antibody (MAb) 60.3* (lower panel). *For clarity, relative amounts of eosinophils and basophils (completing the columns to 100%) were omitted; their percentages did not differ over time or with treatment. Values represent means of five animals in each experimental group with error bars indicating SD. In the case of monocytes, error bars were not inserted for reasons of clarity. PMNs, polymorphonuclear neutrophils.*

that the described rabbit homologue of VCAM-1 would account for the CD18-independent portion of mononuclear cell adherence in our model.

Regarding the effects of the inhibited leukocyte invasion on the development of intimal thickening, especially on SMC migration, no causal connection was detected between PMN infiltration and SMC migration into the intima. PMNs have also been described in other models of experimental intimal thickening or atherosclerotic lesions,[6,13,16,17,25,26] but their functional significance was a matter of speculation. In addition to the putative role in remodeling the arterial wall,[25] they were reported to have the potential to stimulate SMC proliferation.[27] Whether PMNs exert this proliferation-promoting function in our model, thereby contributing to intimal thickening, requires further investigation. However, a triggering effect of PMNs on SMC migration into the intima can be excluded by our results.

The fact that SMCs occurred within the intima of the MAb 60.3–treated rabbits, despite the reduced accumulation of mononuclear cells, leaves open the question of what role mononuclear cells play in SMC migration. From our results we can only conclude that processes that induce SMC movement toward the intima are sufficiently stimulated, although mononuclear cell infiltration is diminished. Monocytes/macrophages, frequently observed in close association with SMCs that penetrate pores of the internal elastic lamina in the early lesions of our model, could provide mediators for SMC migration. A secretory product of monocytes/macrophages, platelet-derived growth factor, for instance, was shown to have chemotactic activity for SMCs.[24,61–63] Other mechanisms, however, may also be involved in regulating the migratory behavior of SMCs (e.g., releasing chemoattractants from ECs[64,65] or generating tissue-type plasminogen activator[66]). Tools capable of eliminating the CD18-independent portion of mononuclear cell emigration will help elucidate the contribution of these cells to SMC migration.

In conclusion, the model of the ES rabbit carotid artery has proven to be suitable for investigating 1) the mechanisms underlying the enhanced recruitment of leukocytes during intimal thickening formation and 2) the link between leukocytes that invade the arterial intima and vascular SMC migration. Essential prerequisites for this study were the presence of a morphologically intact endothelium and the speed of SMC migration into the intima, which proved even faster than after balloon catheter injury.[67] Present work provides evidence that the leukocyte adhesion complex CD18, recognized by MAb 60.3 and in conjunction with the corresponding endothelial counterreceptors, accounts for both neutrophil and mononuclear cell emigration. For mononuclear leukocytes, however, an inhibited extravasation with CD18 MAb was only partly achieved, indicating the involvement of additional receptor–ligand systems. The fact that complete elimination of PMN emigration failed to prevent SMC migration excludes PMNs as initiators of this key event in the development of intimal lesions. This finding adds new information to the understanding of SMC behavior in experimental intimal thickening and, possibly, in atherogenesis.

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References

1. Saphir O, Gore I: Evidence for an inflammatory basis of coronary arteriosclerosis in the young. *Arch Pathol Lab Med* 1950;49: 418–426
2. Greditzer HG, Fischer VW: A sequential ultrastructural study of different arteries in the hypertensive rat. *Exp Med Pathol* 1978;29: 12–28
3. Massmann J, Jellinek H: Hematogenic cell infiltration of the aortic intima in normal and hypercholesterolemic swine: Studies on en face endothelium–intima preparations. *Exp Pathol* 1980;18:11–24
4. Gerrity RG: The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am J Pathol* 1981;103:181–190
5. Joris J, Zand T, Nunnari JJ, Krolikowski FJ, Majno G: Studies on the pathogenesis of atherosclerosis: I. Adhesion and emigration of

mononuclear cells in the aorta of hypercholesterolemic rats. *Am J Pathol* 1983;113:341–358
6. Klurfeld DM: Interactions of immune function with lipids and atherosclerosis. *CRC Crit Rev Toxicol* 1983;11:333–365
7. Faggiotto A, Ross R, Harker E: Studies of hypercholesterolemia in the nonhuman primate: I. Changes that lead to fatty streak formation. *Arteriosclerosis* 1984;4:323–340
8. Schwartz CJ, Sprague EA, Kelly JL, Valente AJ, Suenram CA: Aortic intimal monocyte recruitment in the normo and hypercholesterolemic baboon *(Papio cynocephalus):* An ultrastructural study: Implications in atherogenesis. *Virchows Arch [A]* 1985;405: 175–191
9. Aqel NM, Ball RY, Waldmann H, Mitchinson MJ: Identification of macrophages and smooth muscle cells in human atherosclerosis using monoclonal antibodies. *Am J Pathol* 1985;146:197–204
10. Stary HC: Macrophages, macrophage foam cell, and eccentric intimal thickening in the coronary arteries of young children. *Atherosclerosis* 1987;64:91–107
11. Emeson EE, Robertson AL: T lymphocytes in aortic and coronar intimas: Their potential role in atherogenesis. *Am J Pathol* 1988; 130:369–376
12. Verheyen AK, Vlaminckx EM, Lauwers FM, Saint-Guillain ML, Borgers MJ: Identification of macrophages in intimal thickening of rat carotid arteries by cytochemical localization of purine nucleoside phosphorylase. *Arteriosclerosis* 1988;8:759–767
13. Prescott MF, Karboski McBride C, Court M: Development of intimal lesions after leukocyte migration into the vascular wall. *Am J Pathol* 1989;135:835–846
14. Stary HC: The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. *Eur Heart J* 1990;11(suppl E):3–19
15. Still WJS: The effect of chronic hypertension on the aortic intima of the rat. *Exp Mol Pathol* 1979;31:1–9
16. Stary HC, Malinow MR: Ultrastructure of experimental coronary artery atherosclerosis in cynomolgus macaques. *Atherosclerosis* 1982;43:151–175
17. Faggiotto A, Ross R: Studies of hypercholesterolemia in the nonhuman primate: II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis* 1984;4:341–356
18. Gown AM, Tsukada T, Ross R: Human atherosclerosis: II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am J Pathol* 1986;125:191–207
19. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK: Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 1986;6:131–138
20. Hansson GK, Jonasson L, Lojsthed B, Stemme S, Kocher O, Gabbiani G: Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques. *Atherosclerosis* 1988;72:135–141
21. Munro JM, Cotran RS: The pathogenesis of atherosclerosis: Atherogenesis and inflammation. *Lab Invest* 1988;58:249–261
22. Watanabe T, Tokunaga O, Fan J, Shimokama T: Atherosclerosis and macrophages. *Acta Pathol Jpn* 1989;39:473–486
23. Hansson GK, Jonasson L, Seifert PS, Stemme S: Immune mechanisms in atherosclerosis. *Arteriosclerosis* 1989;9:567–578
24. Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden LT, Masuko H, Sato H: Localization of PDGF-B protein in macrophages in all phases of atherogenesis. *Science* 1990;248:1009–1012
25. Trillo AA: The cell population of aortic fatty streaks in African green monkey with special reference to granulocytic cells: An ultrastructural study. *Atherosclerosis* 1982;43:253–275
26. Cole CW, Hagen PO, Lucas JF, Mikat EM, O'Malley MK, Radic ZS, Makhoul RG, McCann RL: Association of polymorphonuclear leukocytes with sites of aortic catheter-induced injury in rabbits. *Atherosclerosis* 1987;67:229–236
27. Cole CW, Makhoul RG, McCann RL, O'Malley MK, Hagen PO: Vascular smooth muscle cells proliferate in vitro in response to human polymorphonuclear leukocytes and indomethacin. (abstract) *Clin Invest Med* 1985;8:228
28. Betz E, Schlote W: Responses of vessel walls to chronically applied electrical stimuli. *Basic Res Cardiol* 1979;74:10–20
29. Kling D, Holzschuh T, Betz E: Temporal sequence of morphological alterations in artery walls during experimental atherogenesis: Occurrence of leukocytes. *Res Exp Med* 1987;187:237–250
30. Kling D, Holzschuh T, Strohschneider T, Betz E: Enhanced endothelial permeability and invasion of leukocytes into the artery wall as initial events in experimental arteriosclerosis. *Int Angiol* 1987;6: 21–28
31. Grünwald J, Fingerle J, Hämmerle H, Betz E, Haudenschild CC: Cytocontractile structures and proteins of smooth muscle cells during the formation of experimental lesions. *Exp Med Pathol* 1987;46:78–88
32. Huth F, Kojimahara M, Franken T, Rhedin P, Rosenbauer KA: Aortic alterations in rabbits following sheathing with silastic and polyethylene tubes. *Curr Top Pathol* 1975;60:1–32
33. Hirosumi J, Nomoto A, Ohkubo Y, Sekiguchi C, Mutoh S, Yamaguchi I, Aoki H: Inflammatory responses in cuff-induced atherosclerosis in rabbits. *Atherosclerosis* 1987;64:243–254
34. Booth RFG, Martin JF, Honey AC, Hassall DG, Beesley JE, Moncada S: Rapid development of atherosclerotic lesions in the rabbit carotid artery induced by perivascular manipulation. *Atherosclerosis* 1989;76:257–268
35. Heinle H: Metabolite concentration gradients in the arterial wall of experimental atherosclerosis. *Exp Med Pathol* 1987;46:312–320
36. Carlos TM, Harlan JM: Membrane proteins involved in phagocyte adherence to endothelium. *Immunol Rev* 1990;114:5–28
37. Harlan JM, Killen PD, Senecal FM, Schwartz BR, Yee EK, Taylor RF, Beatty PG, Price TH, Ochs HD: The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro. *Blood* 1985;66:167–178
38. Patarroyo M, Makgoba W: Leukocyte adhesion to cells: Molecular basis, physiological relevance and abnormalities. *Scand J Immunol* 1989;30:129–164
39. Beatty PG, Ledbetter JA, Martin PJ, Price TH, Hansen JA: Definition of a common leukocyte cell-surface antigen (Lp95–150) associated with diverse cell-mediated immune functions. *J Immunol* 1983;131:2913–2918
40. Price TH, Beatty PG, Corpuz SR: In vivo inhibition of neutrophil function in the rabbit using monoclonal antibody to CD18. *J Immunol* 1987;139:4174–4177
41. Wetzel BK, Horn RG, Spicer SS: Fine structural studies on the development of heterophil, eosinophil, and basophil granulocytes in rabbits. *Lab Invest* 1967;16:349–380
42. Tanaka Y, Goodman JR: *Electron Microscopy of Human Blood Cells.* New York, Harper and Row Publishers, Inc, 1972
43. McCullagh P, Nelder JA: *Generalized Linear Models.* London, Chapman and Hall, 1989
44. Wallis WJ, Hickstein DD, Schwartz BR, June CH, Ochs HD, Beatty PG, Klebanoff SJ, Harlan JM: Monoclonal antibody-defined functional epitopes on the adhesion-promoting glycoprotein complex (CDw18) of human neutrophils. *Blood* 1986;67: 1007–1013
45. Vedder NB, Winn RK, Rice CL, Chi EY, Arfors KE, Harlan JM: A monoclonal antibody to the adherence-promoting leukocyte glycoprotein, CD18, reduces organ injury and improves survival from hemorrhagic shock and resuscitation in rabbits. *J Clin Invest* 1988; 81:939–944
46. Minick CR, Murphy GE: Experimental induction of arteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet: II. Effect of repeatedly injected foreign protein in rabbits fed a lipid-rich cholesterol-poor diet. *Am J Pathol* 1973;73:265–300
47. Arfors KE, Lundberg C, Lindbom L, Lundberg K, Beatty PG, Harlan JM: A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood* 1987;69:338–340
48. Kuijpers TW, Roos D: Leukocyte membrane adhesion proteins LFA-1, CR3 and p150,95: A review of functional and regulatory aspects. *Res Immunol* 1989;140:461–486
49. Patarroyo M, Prieto J, Rincon J, Timonen T, Lundberg C, Lindbom L, Åsjö B, Gahmberg CG: Leukocyte-cell adhesion: A molecular process fundamental in leukocyte physiology. *Immunol Rev* 1990;114:67–108
50. Springer TA: Adhesion receptors of the immune system. *Nature* 1990;346:425–434
51. Robinson KR: The responses of cells to electrical fields: A review. *J Cell Biol* 1985;101:2023–2027
52. Eitel W, Schmid G, Schlote W, Betz E: Early arteriosclerotic changes of the carotid artery wall induced by electrostimulation. *Pathol Res Pract* 1980;170:211–229
53. Kling D, Heinle H, Harlan JM: Participation of leukocytes in the development of experimentally induced arteriosclerotic lesions: Morphological and functional aspects, in Hauss WH, Wissler RW, Bauch HJ (eds): *New Aspects of Metabolism and Behaviour of Mesenchymal Cells During the Pathogenesis of Arteriosclerosis: Proceedings of the Sixth Münster International Arteriosclerosis Symposium.* Opladen, FRG, Westdeutscher Verlag, 1991, pp 105–115

54. Bochner BS, Luscinskas FW, Gimbrone MA, Newman W, Sterbinsky SA, Derse-Anthony CP, Klunk D, Schleimer RP: Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1–activated human vascular endothelial cells: Contributions of endothelial cell adhesion molecules. *J Exp Med* 1991;173: 1553–1557
55. Walsh GM, Mermod JJ, Hartnell A, Kay AB, Wardlaw AJ: Human eosinophil, but not neutrophil, adherence to IL-1–stimulated human umbilical vascular endothelial cells is $\alpha_4\beta_1$ (very late antigen–4) dependent. *J Immunol* 1991;146:3419–3423
56. Hakkert BC, Kuijpers TW, Leeuwenberg JFM, van Mourik JA, Roos D: Neutrophil and monocyte adherence to and migration across monolayers of cytokine-activated endothelial cells: The contribution of CD18, ELAM-1, and VLA-4. *Blood* 1991;78: 2721–2726
57. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR: VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/ fibronectin binding site. *Cell* 1990;60:577–584
58. Schwartz BR, Wayner EA, Carlos TM, Ochs HD, Harlan JM: Identification of surface proteins mediating adherence of CD11/ CD18-deficient lymphoblastoid cells to cultured human endothelium. *J Clin Invest* 1990;85:2019–2022
59. Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosso M, Osborn L, Newman B, Lobb RR, Harlan JM: Vascular cell adhesion molecule–1 mediates lymphocyte adherence to cytokine activated cultured endothelial cells. *Blood* 1990;76:965–970
60. Cybulsky MI, Gimbrone MA: Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 1991;261:788–791
61. Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross R: A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell* 1985;43:277–286
62. Martinet Y, Bitterman PB, Mornex JF, Grotendorst GR, Martin GR, Crystal RG: Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. *Nature* 1986;319:158–160
63. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW: Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest* 1992;89:507–511
64. DiCorleto PE, Chisolm GM: Participation of the endothelium in the development of the atherosclerotic plaque. *Prog Lipid Res* 1986;25:365–374
65. Zerwes HG, Risau W: Polarized secretion of a platelet-derived growth factor–like chemotactic factor by endothelial cells in vitro. *J Cell Biol* 1987;105:2037–2041
66. Clowes AW, Clowes MM, Au YPT, Reidy MA, Belin D: Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circ Res* 1990;67:61–67
67. Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury: I. Smooth muscle growth in the absence of endothelium. *Lab Invest* 1983;49:327–333

29. M. Peeters *et al.*, *ibid.* 3, 625 (1989).
30. T. Huet, R. Cheynier, A. Meyerhans, G. Roelants, S. Wain-Hobson, *Nature* 345, 356 (1990).
31. V. M. Hirsch *et al.*, *ibid.* 339, 389 (1989).
32. R. F. Doolittle, D. F. Feng, M. S. Johnson, M. A. McClure, *Q. Rev. Biol.* 64, 1 (1989).
33. G. Myers *et al.*, Eds., *Human Retroviruses and AIDS 1989, A Compilation and Analysis of Nucleic and Amino Acid Sequence* (Los Alamos National Laboratory, Los Alamos, NM, 1989).
34. R. M. Anderson and R. M. May, *Sci. Am.* 266, 58 (May 1992).
35. ———, M. C. Boily, G. P. Garnett, J. T. Rowley, *Nature* 352, 581 (1991).
36. R. M. Krause, *Rev. Infect. Dis.* 6, 270 (1984).
37. S. O. Aral and K. K. Holmes, *Sci. Am.* 264, 62 (February 1991).
38. M. Burnet, *Natural History of Infectious Diseases* (Cambridge Univ. Press, London, ed. 3, 1963).
39. *NIH Data Books 1989 and 1991* (NIH Publ. 89-1261 and 91-1261, Department of Health and Human Services, NIH, Washington, DC, 1989 and 1991).
40. R. M. Krause, *The Restless Tide: The Persistent Challenge of the Microbial World* (The National Foundation for Infectious Diseases, Washington, DC, 1981).
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Structure-Based Strategies for Drug Design and Discovery

Irwin D. Kuntz

Most drugs have been discovered in random screens or by exploiting information about macromolecular receptors. One source of this information is in the structures of critical proteins and nucleic acids. The structure-based approach to design couples this information with specialized computer programs to propose novel enzyme inhibitors and other therapeutic agents. Iterated design cycles have produced compounds now in clinical trials. The combination of molecular structure determination and computation is emerging as an important tool for drug development. These ideas will be applied to acquired immunodeficiency syndrome (AIDS) and bacterial drug resistance.

Will the next generation of pharmaceuticals arise from a combination of crystallography and computational methods (*1–5*)? While I share the enthusiasm for structure-based drug design (*6–8*), it is the newest of several approaches to the lengthy process of finding and developing therapeutic agents (Table 1). One important discovery procedure is high-volume "random" screening of natural products, corporate databases of compounds, or peptides and oligonucleotides. Another method is the interception of specific biochemical mechanisms. Vaccine development is yet another route to anti-infectives. Finally, there are well-developed "active analog" approaches to improve upon initial discoveries. Any of these techniques, singly or in combination, can play a pivotal role in finding new drugs.

Can we design drugs from first principles, creating a molecule with a specific mode of action and acceptable biological properties? Today's answer is "no." What we can reliably expect is to design inhibitors, especially enzyme inhibitors, and to begin the long process of drug development from a sensible starting point.

Fifteen years ago, Seymour Cohen proposed a general paradigm for developing drugs for infectious diseases (*9*). Infectious agents, such as viruses, bacteria, fungi, or protozoa, encode or carry their own crucial enzymes and nucleic acids, which serve as obvious targets for intervention. In the succeeding decade and a half, the ability to identify, clone, express, and purify proteins and nucleic acids has increased enormously, making highly specific in vitro assay systems commonplace. These assays, in turn, lead to effective strategies for the discovery of a wide variety of inhibitors (*10*). Structural techniques have also advanced, and high-resolution molecular anatomies can be determined by crystallographic and magnetic resonance experiments. Thus the pieces are in place to extend Cohen's concept to structure-based design (*1, 3, 7, 11, 12*).

I will draw examples from AIDS and bacterial drug resistance. These two major health problems have three features in common: recent starting points as public health issues, known etiologies, and a large number of macromolecules as potential targets (Table 2).

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Screening

The vast majority of drugs in the marketplace were derived from discoveries in large-scale screens or from analog development programs. Robotic systems can perform thousands of tests per day by means of radioactive labeling or spectroscopic detection, and further improvements can be expected (*13*). It is feasible to scan an entire corporate database (for example, 100,000 to 500,000 compounds) in less than a year's time. The coupling of cell metabolism to microsensors opens the door to rapid surveys of toxicity and function at the cellular level (*14*). The only current approved drugs against human immunodeficiency virus (HIV) were detected with screening techniques (*15*) and so were the original generation of antibiotics.

Understanding the biological or biochemical mechanism of a disease often suggests the types of molecules needed for new drugs (*16, 17*). Examples are substrate or cofactor analogs for thymidylate synthase as antitumor agents (*18, 19*) or the development of the captopril family of antihypertensives (*17*). In a similar manner, clavulinic acid acts as a β-lactamase inhibitor (*20*). Such efforts represent a proven route from test tube to pharmacy.

Substrate-Based Design of Protease Inhibitors

There are circumstances in which the "rational" design of inhibitors can be performed without a target structure. A good example is a two-step protocol for developing protease inhibitors: (i) characterize the substrate specificities of the protease; and (ii) synthesize peptides with similar features but with the hydrolyzable amide bond replaced by a nonreactive "isostere." The peptides can subsequently be optimized by modifications in the side chains or backbone. This approach has been used for renin inhibitors (*21*) and for inhibitors of the HIV-1 protease (*22*). One can proceed further by adding specific moieties such as chloromethyl ketones or phosphonates that are capable of forming transition-state analog complexes with the enzyme. Among the examples are inhibitors of the *Schistosoma mansoni* cercarial elastase (*23*) and carboxypeptidase A (*24*). It is reasonable to expect to obtain peptidelike inhibitors with nanomolar inhibitory constants in in vitro assays after 1 year of effort.

Table 1. Drug development steps (*71*).

Step	Years
Discovery and lead generation	1–2
Lead optimization	1–2
In vitro and in vivo assays	1–2
Toxicology trials	1–3
Human safety trials	1
Human efficacy trials	1–2
Total development time	6–12

The design of protease in ors provides a directed and logical progression. This strategy is greatly assisted by the recent dramatic improvements in peptide synthesis and in screening by chemical or biological means (25–28). However, for many applications, there are problems with peptide-like agents as drugs (29). Frequently, they have short biological half-lives and poor bioavailability. Binding kinetics must also be considered (30). Hydrolysis can be reduced with D-amino acids or additional backbone modifications, but rapid clearance remains a general problem for molecular sizes above 800 to 1000 daltons. The prospects for oral delivery of peptides are uncertain, but injectable formulations are readily accessible, and other delivery modes—such as inhalation, nasal absorption, or electroporation—are under active study (31). The conversion of a peptide-based inhibitor into an orally active drug is an important challenge for the field of synthetic chemistry. There is no general solution to this "peptidomimetic" problem, but efforts include modification of the amide backbone (32), cyclization (33), β-turn mimics (34), and the use of unusual amino acids (35). Analogous issues arise in the use of oligonucleotides as therapeutic agents (36, 37).

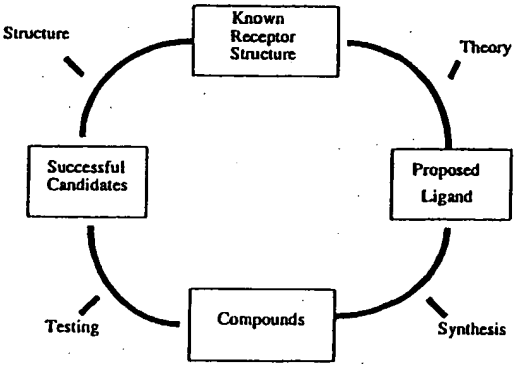
Computational Strategies

Computer-based techniques can assist in both the discovery and the optimization of lead compounds. Macromolecular structures are useful in this effort, but they are not required if families of active compounds are available. In this seminal work, Hansch examined quantitative structure-activity relationships (QSAR) between biological activity and the underlying chemical properties such as atomic charges, oil-water partition coefficients, and molecular volumes (38). Extensions of the approach to three-dimensional (3-D) representation. e available (39, 40). Alternatively, large databases of compounds can be searched for molecules with chemical or structural similarity to active leads. These methods have had relatively little impact on AIDS research to this point, but they have played a role in the development of sulfanilamide and cephalosporin antibodies (41, 42).

Structure-Based Design

The central assumption of structure-based design is that good inhibitors must possess significant structural and chemical complementarity to their target receptor (43). A four-step cycle for combining structural information and computational efforts is illustrated in Fig. 1. A structure of any form of the receptor provides a starting point for direct modeling activities. The structures of ligand-receptor complexes or of homologous receptors also contain valuable information. Repeated application of the cycle of Fig. 1 has led to compounds in clinical trials (3, 4). Structural insight is enhanced by a variety of computer programs, ranging from interactive designs with computer graphs to automated database searching (44) (Table 3). Several biotechnology companies have been formed primarily to carry out structure-based design, and most major pharmaceutical companies have structural and computational groups as part of their drug discovery effort.

Our own experience in the discovery of lead compounds illustrates this partnership of structure determination and computational efforts. We have developed a computer program called DOCK (45) that is used to solve the 3-D jigsaw puzzle of fitting putative "ligands" into appropriate sites on the receptor. A starting point is an x-ray crystallographic structure of the macromolecule. High-quality model-built structures, based on homologous proteins, are also proving useful (23). DOCK explores three important aspects of drug discovery: creation of a negative image of the target site, placement of the putative "ligands" into the site, and evaluation of the quality of fit (12, 45–47).



Design Paradigm

Fig. 1. General approach to the structure-based design of biological inhibitors. Begin with the determination of the structure of the target receptor. Theoretical principles and experimental data are used to propose a series of putative ligands. These compounds are synthesized and tested. The final step is the determination of the structure of the receptor-ligand complex. The figure emphasizes the cyclic and multidisciplinary aspects of this type of project.

Table 2. Macromolecular targets (**A**) for inhibition of HIV (*72, 73*) and (**B**) for drug-resistant bacteria.

	Target	Function	Intervention	Structures
A	CD4	Human cell recognition site	Vaccines, soluble CD4	X-ray (*74, 75*)
	gp120	Viral protein that recognizes CD4	Vaccines, soluble CD4	
	p24	Capsid stability		
	Reverse transcriptase	Converts viral RNA into DNA	AZT, ddC, ddI	X-ray (*76, 77*)
	RNA-DNA	Transcription intermediate	RNA, DNA ligands	Model
	RNase H	Removes viral RNA	RNase inhibitors	X-ray (*78–81*) NMR (*82, 83*)
	Integrase	Incorporates DNA into host genome		
	tat	Regulates viral transcription	Benzodiazepines, nucleosides	NMR (*84*)
	TAR	tat binding region	Antisense oligonucleotides	
	rev	Trans-activating factor		
	Protease	Processes viral polyprotein	Protease inhibitors	X-ray (*85–92*)
B	Dihyropteroate synthase	Folate pathway	Sulphonamides	
	Dihydrolfolate reductase	Folate pathway	Trimethoprim	X-ray (*93, 94*)
	β-lactamase	Hydrolyzes lactams	Clavulinic acid	X-ray (*95, 96*)
	DNA gyrase	DNA supercoiling	4-Quinolones	X-ray (*97*)
	Aminoglycoside modifying enzymes	Chemical model of antibiotics		

As a first step, DOCK characterizes the entire surface of the macromolecule, seeking the grooves and invaginations in the surface that form the target sites. These sites are filled with sets of overlapping spheres. A set of sphere centers serves as the negative image of a specific site (Fig. 2). Typically, the sites found by the program include the active regions of enzymes, recognition and allosteric features, and other small pockets that have no known function (45). The program is not restricted to examining enzymes. It has been applied to nucleic acid structures (48, 49), viral coat proteins (50), and the study of protein-protein binding interactions (51). Automatic characterization of potential binding sites is especially useful in examining complex viral surface proteins such as hemagglutinin (50). We can also use a "positive image" of a macromolecular surface by reversing the mathematical procedure and producing spheres inside the "receptor" (51).

The second step in the DOCK algorithm matches x-ray or computer-derived structures of putative ligands (52) to the image of the receptor on the basis of a comparison of internal distances. Matching algorithms come from a well-studied area in combinatorial mathematics called the "isomorphic subgraph" problem (53). Although a systematic search of all ways to fit two objects together is not feasible, rapid heuristic approximations are available (45, 46, 51) that examine thousands of alternative geometric matches per second. Each of these orientations must be evaluated to measure the goodness of fit of the "ligand" to the site. At first, we used a simple proximity scoring method (12, 45, 54) as a measure of steric complementarity. Recently, we have expanded the scoring functions to include a full intermolecular force field (47).

The program searches 3-D databases of small molecules and ranks each candidate on the basis of the best orientations that can be found for a particular molecular conformation (12, 46, 47). Each molecule can be evaluated either on its own merits or as a template. The template concept encourages chemists to look beyond the literal database entries to the design of new chemical species. The ability of DOCK to propose specific molecules in specific orientations in the active site is one of its strongest features. Although some of these molecules are related to substrates, cofactors, or known inhibitors, others can be of novel structures (12).

Computer programs such as DOCK can provide a rapid and controlled exploration of the geometric intricacies of target sites. Ligands can be examined at a rate of 10 to 100 per minute, making it possible to examine databases of 100,000 compounds in less than a week with a workstation. Implementing the program on a supercomputer or parallel-processing device makes it possible to search a corporate database of 500,000 compounds in a day.

Databases of interest for drug design include the Cambridge Structural Database (CSD), a compendium of approximately 100,000 molecules whose crystal structures have been determined (55), the Fine Chemicals Directory (FCD) distributed by Molecular Design Limited (San Leandro, California), and, in prototype form, portions of the Chemical Abstracts registry. The latter two databases have been generated in 3-D form by means of a rule-based conformation generator called CONCORD, developed by R. Pearlman at the University of Texas (52). Most corporate databases have been converted into 3-D coordinates by CONCORD.

Using DOCK and the CSD and FCD databases, my colleagues have found or designed inhibitors for a wide variety of enzymatic and receptor systems (Table 4). Typically, the 100 to 200 best-scoring compounds are examined with computer graphics (Table 3). Of these, 10 to 50 are selected for testing on the basis of chemical and toxicological properties. We find that between 2 and 20% of the compounds tested show inhibition in the micromolar range. In every case we have tried, DOCK has proven extremely valuable as a computer screening procedure and as a method of generating structural hypotheses about ligand-receptor interactions.

The use of DOCK to optimize leads has been more problematic. The two major difficulties are obtaining the proper ligand

Table 3. Examples of available computer programs (44).

Structure-activity relationships	(38, 98–100)
Graphics	
Interactive graphics	(101)
Molecular surfaces	(102)
Volume rendering	(103)
Molecular calculations	
Quantum mechanics	(104)
Conformation generation	
Systematic	(105–107)
Heuristic	(52)
Distance geometry	(108)
Molecular mechanisms	(109, 110)
Molecular dynamics	(111)
Free energy perturbation	(64, 65)
Docking	(45, 112–117)
Similarity	(118, 119)

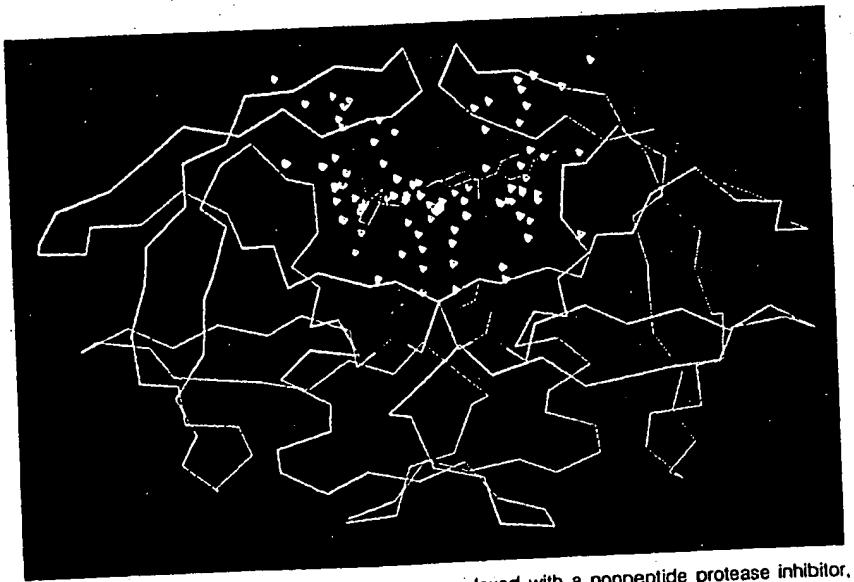


Fig. 2. New structure of HIV-protease (cyan), complexed with a nonpeptide protease inhibitor, UCSF8 (magenta) (120). The negative image of the enzyme active site created by DOCK is shown in yellow. At the bottom of the figure, in red, are the active aspartic acids.

Table 4. DOCK leads developed at the University of California, San Francisco.

System	Affinities		Reference
	1st lead	2nd generation	
HIV protease	100 μM	5 μM	(54, 120)
B-form DNA	10 μM		(49)
RNase H	500 μM		(121)
Thymidylate synthase	900 μM	3 μM	(56)
Hemagglutinin	100 μM	5 μM	(50)
CD4-gp120*	5 μM		(122)
Malaria protease†	10 μM		(123)

*Developed in collaboration with Procept, Inc., Cambridge, Massachusetts. †Structure obtained from homology model-building.

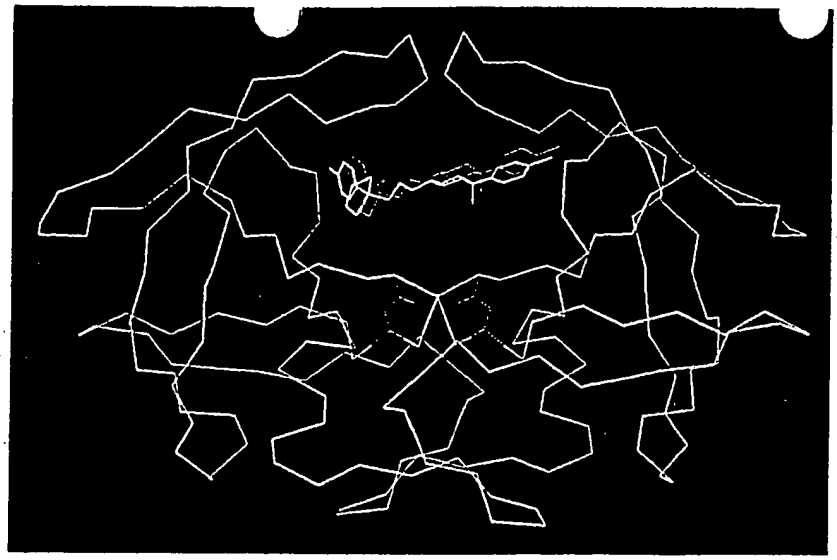


Fig. 3. Observed position (magenta) (*120*) and calculated position (yellow) (*124*) of UCSF 8 with the intermolecular force field scoring procedure (*47*).

conformation and discriminating among several proposed interaction modes of similar energy (*47, 51*). Many assumptions are required to scan large databases in a reasonable amount of time. These include: rigid ligands and rigid receptors, neglect of bound water molecules and counterions, and simplified evaluations of interaction energies. Some of these limitations are being removed as computational power increases. In recent work, with improved force fields and an experimental ligand conformation, the highest scoring orientations produced by DOCK correspond to the crystallographic binding mode within 1 to 2 Å (Fig. 3). In a project to design thymidylate synthase inhibitors, we have proceeded rapidly to affinities of 3 to 5 μM by combining the consensus results from DOCK with crystal structures of a weak inhibitor and a similarity search of the FCD with MACCS software (Molecular Design Limited, San Leandro, California) (*56, 57*).

In sum, DOCK works well as a computer screening procedure for generating leads. To improve the program, we are examining ways to search the conformational space of ligands (*58*) and to correct for desolvation of the ligand and receptor surfaces on binding (*59, 60*). We are also developing a program for interactive docking and design (*61*) and a program to focus on the subtle structural and chemical differences among closely related enzymes (*62*). Docking methods are being explored in several other laboratories (Table 3).

Prediction of accurate free energies of interaction and accurate binding geometries remains an important goal for all structure-based efforts. A promising technique is the free energy perturbation calculation. Surprisingly accurate free energy differences (with 1 to 2 kcal/mol) can be obtained in favorable cases (*63–65*). The method can also include the effects of desolvation. The difficulties with this approach are similar to those described above—it requires good sampling of the conformational and configurational states available to both ligand and receptor. For any computational method, relative accuracies within 1 kcal/mol are required if one seeks quantitative predictions of binding affinities for a series of related compounds. Experiment plays a decisive role in calibrating such efforts.

Response Time of the Drug Design Cycle

The AIDS epidemic and the spread of drug-resistant bacteria illustrate the continued danger posed by infectious diseases. What can be done to shorten the response time of the drug development system? Because the process involves a series of steps, each of approximately the same duration (Table 1), fundamental improvements are needed at every level. Lead discovery can certainly proceed more rapidly with a combination of computer screening, high-volume assays, and more rapid structural determinations. Computer programs can now examine substantial databases in a few days. With improved hardware, 3-D searches of the entire Chemical Abstracts Registry would be feasible. In selected cases, quantitative estimates of binding constants are now on the scientific horizon. The next generation of computers should make such calculations applicable to a wider range of problems. Technical advances continue in the realm of structure determination. High-speed area detectors and the use of synchrotron sources mean that new structures can be completed in a week, and that a turnaround of a structure per day can sometimes be achieved. The least controlled step in crystallography is the growth of crystals. This remains unpredictable and is a serious bottleneck for structure determination for membrane-bound proteins.

Lead optimization and the development of active analogs can move more rapidly in the future if it is possible to adapt the modular chemistry approach that has been so successful in peptide and oligonucleotide synthesis to a wider range of compounds. It should be possible to exploit a core of thoroughly researched general reactions for organic synthesis. It is also crucial to improve toxicological assays by increasing reliance on specially adapted bacterial systems, cell culture, and the use of transgenic animals. Most of the time and money required to develop a drug is spent at the end of the development cycle. The loss of a promising candidate during clinical trials is an expensive and disheartening event. Any procedure that can detect serious obstacles at an early stage is much to be desired.

The immense efforts to find anti-AIDS drugs and to provide effective agents for drug-resistant microorganisms will test the various strategies of drug development. The only drugs useful for HIV available through 1991 were generated by screening known pharmaceuticals. However, several HIV protease inhibitors are moving through clinical trials. These were developed with the substrate-based approach outlined earlier. Nonpeptide inhibitors derived from structure-based efforts are also being reported (*54*). The status of the structure determinations for a number of AIDS-related macromolecules is summarized in Table 2A.

Of specific interest in this issue are the prospects for countering the drug-resistant mechanisms of prokaryotes. The fundamental routes for evasion include enzymatic degradation of drugs, mutation of bacterial target proteins, changes in membrane permeability, and overproduction of key enzymes. Each of these can be attacked through structural efforts. Some targets for bacterial systems are given in Table 2B. The most straightforward efforts involve enzymes such as the β-lactamases or differential inhibition of enzymes on the folate pathway. Other exciting targets deal with drug transport mechanisms (*66, 67*).

Future Prospects

Looking ahead, areas for new work include antiviral, antifungal, and antiparasitic drugs and the problems of general drug resistance

in eukaryotes. Each of these ...as has important targets for structure-based design, for example: the viral coating-uncoating phenomena (68), the mating factor systems in yeast, specific enzymes in parasitic organisms (23, 69), and the multidrug-resistance apparatus in human cells (70). There are encouraging signs that structure-based collaborative projects can have a large impact on these important problems.

REFERENCES AND NOTES

1. L. F. Kuyper *et al.*, *J. Med. Chem.* 28, 303 (1985).
2. T. Abate, in *The Scientist*, p. 13 (13 April 1992)
3. K. Appelt *et al.*, *J. Med. Chem.* 34, 1925 (1991)
4. M. D. Varney *et al.*, *ibid.* 35, 663 (1992).
5. M. A. Navia and M. A. Murko, *Curr. Opin. Struct. Biol.* 2, 202 (1992).
6. C. R. Beddell *et al.*, *Br. J. Pharmacol.* 82, 397 (1984).
7. W. G. J. Hol, *Angew. Chem.* 25, 767 (1986).
8. W. C. Ripka, W. J. Sipio, J. Blaney, *Lect. Heterocycl. Chem.* 9, S95 (1987).
9. S. S. Cohen, *Science* 197, 431 (1977).
10. E. Sarubbi *et al.*, *FEBS Lett.* 279, 265 (1991).
11. C. R. Beddell, P. J. Goodford, F. E. Norrington, S. Wilkinson, R. Wootton, *Br. J. Pharmacol.* 57, 201 (1976).
12. R. DesJarlais *et al.*, *J. Med. Chem.* 31, 722 (1988).
13. R. A. Felder, J. C. Boyd, K. Margrey, W. Holman, J. Savory, *Clin. Chem.* 36, 1534 (1990).
14. J. W. Parce *et al.*, *Science* 246, 243 (1989).
15. H. Mitsuya, *Proc. Natl. Acad. Sci. U.S.A.* 82, 7096 (1985).
16. J. W. Black, W. A. M. Duncan, C. J. Durant, C. R. Ganellin, E. M. Parsons, *Nature* 236, 385 (1972).
17. M. A. Ondetti, B. Rubin, D. W. Cushman, *Science* 196, 441 (1977).
18. D. V. Santi and G. L. Kenyon, in *Burger's Medicinal Chemistry*, M. E. Wolff, Ed. (Wiley-Interscience, New York, 1980), pp. 349–391.
19. T. R. Jones *et al.*, *Eur. J. Cancer* 17, 11 (1981).
20. T. T. Howarth and A. G. Brown, *J. Chem. Soc. Chem. Commun.* 1976, 266 (1976).
21. W. Greenlee, *Pharm. Res.* 4, 364 (1987).
22. C. Debouck, *AIDS Res. Hum. Retroviruses* 8, 153 (1992).
23. F. E. Cohen *et al.*, *Biochemistry* 30, 11221 (1991).
24. A. P. Kaplan and P. A. Bartlett, *ibid.*, p. 8165.
25. H. M. Geysen, S. J. Rodda, T. J. Mason, in *Synthetic Peptides as Antigens*, R. Porter and J. Wheelan, Eds. (Wiley, New York, 1986), pp. 131–149.
26. J. K. Scott and G. P. Smith, *Science* 249, 386 (1990).
27. S. P. A. Fodor *et al.*, *ibid.* 251, 767 (1991).
28. K. S. Lam *et al.*, *Nature* 354, 82 (1991).
29. H. P. Schnebli and N. J. Braun, in *Proteinase Inhibitors*, A. J. Barrett and G. Salvensen, Eds. (Elsevier, New York, 1986), pp. 613–617.
30. J. G. Bieth, *Biochem. Med.* 32, 387 (1984).
31. L. L. Wearley, *Crit. Rev. Ther. Drug Carrier Syst.* 8, 331 (1991).
32. M. Szetke, *Nature* 299, 555 (1982).
33. C. Pattaroni *et al.*, *Int. J. Pep. Protein Res.* 36, 401 (1990).
34. A. C. Bach, J. A. Markwalder, W. C. Ripka, *ibid.* 38, 314 (1991).
35. N. A. Roberts *et al.*, *Science* 248, 358 (1990).
36. M. L. Riordan and J. C. Martin, *Nature* 350, 442 (1991).
37. W. F. Anderson, *Science* 256, 808 (1992).
38. C. Hansch, *Acc. Chem. Res.* 2, 323 (1969).
39. R. D. Cramer, D. E. Patterson, J. D. Bunce, *Prog. Clin. Biol. Res.* 29, 161 (1989).
40. G. M. Crippen, *J. Med. Chem.* 23, 599 (1980).
41. P. G. De Benedetti *et al.*, *ibid.* 30, 459 (1987).
42. J. Pfeil-Doyle, S. E. Draheim, S. Kukolja, J. L. Ott, F. T. Counter, *ibid.* 31, 1993 (1988).
43. P. M. Dean, *Molecular Foundations of Drug-Receptor Interactions* (Cambridge Univ... s, Cambridge, 1987).
44. N. C. Cohen, J. M. Blaney, C. Humblet, P. Gund, D. C. Barry, *J. Med. Chem.* 33, 883 (1990).
45. I. D. Kuntz, J. M. Blaney, S. J. Oatley, R. Langridge, T. E. Ferrin, *J. Mol. Biol.* 161, 269 (1982).
46. B. K. Shoichet, D. L. Bodian, I. D. Kuntz, *J. Comp. Chem.* 13, 380 (1992).
47. E. C. Meng, B. K. Shoichet, I. D. Kuntz, *ibid.*, p. 505.
48. P. D. J. Grootenhuis, P. A. Kollman, G. L. Seibel, R. L. DesJarlais, I. D. Kuntz, *Anti-Cancer Drug Des.* 5, 237 (1990).
49. S. M. Kerwin, I. D. Kuntz, G. L. Kenyon, *Med. Chem. Res.* 1, 361 (1991).
50. D. Bodian *et al.*, in preparation.
51. B. K. Shoichet and I. D. Kuntz, *J. Mol. Biol.* 221, 327 (1991).
52. A. Rusinko *et al.*, *J. Chem. Inf. Comput. Sci.* 29, 251 (1989).
53. R. J. Wilson, *Introduction to Graph Theory* (Longman, London, 1972).
54. R. L. DesJarlais *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87, 6644 (1990).
55. F. H. Allen *et al.*, *Acta Crytallogr. Sect. B* 35, 2331 (1979).
56. B. K. Shoichet, I. D. Kuntz, D. Santi, K. Perry, R. Stroud, in preparation.
57. K. Perry, R. Stroud, B. Shoichet, I. D. Kuntz, in preparation.
58. A. R. Leach and I. D. Kuntz, *J. Comp. Chem.* 13, 730 (1992).
59. A. A. Rashin and M. A. Bukatin, *J. Phys. Chem.* 95, 2942 (1991).
60. W. C. Still, A. Tempczyk, R. C. Hawley, T. Hendrickson, *J. Am. Chem. Soc.* 112, 6127 (1990).
61. R. A. Lewis *et al.*, *J. Mol. Graphics* 10, 66 (1992).
62. D. Bodian and I. D. Kuntz, unpublished results.
63. K. Merz and P. A. Kollman, *J. Am. Chem. Soc.* 111, 5649 (1989).
64. D. L. Beveridge and F. M. DiCapua, *Annu. Rev. Biophys. Biophys. Chem.* 18, 431 (1989).
65. W. F. van Gunsteren and H. J. C. Berendsen, *Angew. Chem. Int. Ed. Engl.* 29, 992 (1990).
66. J. A. Endicott and V. Ling, *Annu. Rev. Biochem.* 58, 137 (1989).
67. J. I. Ross *et al.*, *Mol. Microbiol.* 4, 1207 (1990).
68. T. J. Smith *et al.*, *Science* 233, 1286 (1986).
69. U. Edman, J. C. Edman, B. Lundgren, D. V. Santi, *Proc. Natl. Acad. Sci. U.S.A.* 86, 6503. (1989).
70. I. C. West, *Trends Biochem. Sci.* 15, 42 (1990).
71. J. A. Dimasi, N. R. Bryant, L. Lasagna, *Clin. Pharmacol. Ther.* 50, 471 (1991).
72. H. Mitsuya, R. Yarchoan, S. Broder, *Science* 249, 1533 (1990).
73. M. Stevenson, M. Bukrinsky, S. Haggerty, *AIDS Res. Hum. Retroviruses* 8, 107 (1992).
74. J. H. Wang *et al.*, *Nature* 348, 411 (1990).
75. S. E. Ryu *et al.*, *ibid.*, p. 419.
76. E. W. Arnold *et al.*, *ibid.* 357, 85 (1992).
77. L. A. Kohlstaedt, J. Wang, J. M. Friedman, P. A. Rice, T. A. Steitz, *Science* 256, 1783 (1992).
78. W. Yang, W. A. Hendrickson, R. J. Crouch, Y. Satow, *ibid.* 249, 1398 (1990).
79. J. F. I. Davies, Z. Hostomska, Z. Hostomsky, S. R. Jordan, D. A. Matthews, *ibid.* 252, 88 (1991).
80. K. Katayanagi *et al.*, *Nature* 347, 306 (1990).
81. K. Katayanagi *et al.*, *J. Mol. Biol.* 223, 1029 (1992).
82. R. Powers *et al.*, *ibid.* 221, 1081 (1991).
83. T. Yamazaki, M. Yoshida, S. Kanaya, H. Nakamura, K. Nagayama, *Biochemistry* 30, 6036 (1991).
84. M. F. Summers, T. L. South, B. Kim, D. R. Hare, *ibid.* 29, 329 (1990).
85. M. A. Navia *et al.*, *Nature* 337, 615 (1989).
86. A. Wlodawer *et al.*, *Science* 245, 616 (1989).
87. R. Lapatto *et al.*, *Nature* 342, 299 (1989).
88. M. Miller *et al.*, *Science* 246, 1149 (1989).
89. P. M. Fitzgerald *et al.*, *J. Biol. Chem.* 265, 14209 (1990).
90. J. Erickson *et al.*, *Science* 249, 527 (1990).
91. M. Jaskolski *et al.*, *Biochemistry* 30, 1600 (1991).
92. R. Bone, J. P. Vacca, P. S. Anderson, M. K. Holloway, *J. Am. Chem. Soc.* 113, 9382 (1992).
93. D. A. Matthews *et al.*, *Science* 197, 452 (1977)
94. J. T. Bolin, D. J. Filman, D. A. Matthews, R. C. Hamlin, J. Kraut, *J. Biol. Chem* 257, 13650 (1982).
95. C. Oefner *et al.*, *Nature* 343, 284 (1990)
96. O. Herzberg, *J. Mol. Biol.* 217, 701 (1991)
97. D. B. Wigley, G. J. Davies, E. J. Dodson, A. Maxwell, *Nature* 351, 624 (1991)
98. C. Hansch and T. Klein, *Acc. Chem. Res.* 19, 392 (1986).
99. R. D. Cramer III, *J. Am. Chem. Soc.* 110, 5959 (1988).
100. A. K. Ghose and G. M. Crippen, *Mol. Pharm.* 37, 725 (1990).
101. R. Langridge, T. E. Ferrin, I. D. Kuntz, M. L. Connolly, *Science* 211, 661 (1981)
102. M. L. Connolly, *ibid.* 221, 709 (1983)
103. D. S. Goodsell, I. S. Mian, A. J. Olson, *J. Mol. Gr.* 7, 41 (1989).
104. W. J. Hehre, L. Radom, P. v. R. Schleyer, J. A. Pople, *Ab Initio Molecular Orbital Theory* (Wiley, New York, 1986).
105. G. R. Marshall, C. D. Barry, H. E. Bosshard, R. A. Dammkoehler, D. A. Dunn, in *Computer Assisted Drug Design* (American Chemical Society, Washington, DC, 1978), pp. 205–226.
106. D. P. Dolata, A. R. Leach, K. Prout, *J. Comput.-Aided Mol. Des.* 1, 73 (1987).
107. A. R. Leach and K. Prout, *J. Comp. Chem.* 11, 1193 (1990).
108. G. M. Crippen and T. F. Havel, *Distance Geometry and Molecular Conformation* (Wiley, Taunton, England, 1988).
109. U. Burkert and N. L. Allinger, *Molecular Mechanics* (American Chemical Society, Washington, DC, 1982).
110. T. A. Clark, *A Handbook of Computational Chemistry* (Wiley, New York, 1985)
111. J. A. McCammon and S. C. Harvey, *Dynamics of Proteins and Nucleic Acids* (Cambridge Univ. Press, Cambridge, 1987).
112. P. J. Goodford, *J. Med. Chem.* 28, 849 (1985).
113. D. S. Goodsell and A. J. Olson, *Proteins* 8, 195 (1990).
114. F. Jiang and S. H. Kim, *J. Mol. Biol.* 219, 79 (1991).
115. D. G. Mullen and P. A. Bartlett, in *Proceedings of the European Peptide Symposium*, E. Giralt and D. Andrev, Eds. (ESCOM Scientific, Leiden, 1990), pp. 364–365.
116. A. Miranker and M. Karplus, *Proteins* 11, 29 (1991).
117. M. C. Lawrence and P. C. Davis, *ibid* 12, 31 (1992).
118. J. H. Van Drie, D. Weininger, Y. C. Martin, *J. Comput.-Aided Mol. Des.* 3, 225 (1989)
119. M. A. Johnson and G. M. Maggiora, *Concepts and Application of Molecular Similarity* (Wiley, New York, 1990).
120. E. Rutenber *et al.*, in preparation.
121. C. Levinson, G. L. Kenyon, I. D. Kuntz, unpublished results.
122. M. McGregor, F. E. Cohen, I. D. Kuntz, unpublished results.
123. C. Ring *et al.*, in preparation.
124. E. Meng and I. D. Kuntz, unpublished results

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Increased Expression of Neutrophil and Monocyte Adhesion Molecules in Unstable Coronary Artery Disease

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Background. **A rapid increase in leukocyte adhesion to endothelial cells is one of the first events in the acute inflammatory response and in the pathogenesis of vascular diseases. A subgroup of cell surface glycoproteins (the CD11/CD18 complex) play a major role in the leukocyte adhesion process; in particular, the CD11b/CD18 receptor can be upregulated severalfold in response to chemotactic factors. The purpose of this study was to assess whether upmodulation of granulocyte and monocyte CD11b/CD18 receptors takes place during the passage of blood through the coronary tree of patients with clinical manifestations of ischemic heart disease.**

Methods and Results. **Thirty-nine patients who underwent diagnostic coronary arteriography were studied. Group 1 (15 patients) had a clinical diagnosis of unstable angina, group 2 (14 patients) had stable exertional angina, and group 3 (10 patients) had atypical chest pain. Simultaneous sampling from the coronary sinus and aorta was obtained before coronary arteriography. Cell surface receptors were detected by direct immunofluorescence evaluated by flow cytofluorimetry using monoclonal antibodies tagged with fluorescent markers. Leukocytes were stained in unseparated blood to avoid in vitro manipulation that could activate phagocytes. Group 1 and 2 patients had significant coronary artery disease (>50% coronary narrowing in at least one major coronary vessel), whereas group 3 patients had normal coronary arteries. In group 1, granulocytes and monocytes showed a significantly higher expression of the CD11b/CD18 adhesion receptor in the coronary sinus than in the aorta (both $P<.01$), whereas no difference in CD11b/CD18 expression was seen in groups 2 and 3.**

Conclusions. **Patients with unstable angina have an increased expression of granulocyte and monocyte CD11b/CD18 adhesion receptors, indicating that an inflammatory reaction takes place within their coronary tree. Activation of these leukocytes may induce coronary vasoconstriction, favor thrombotic processes, and further activate platelets, thus having potential implications on the pathogenesis of unstable coronary artery disease. (*Circulation* 1993;88:358-363)**

KEY WORDS • phagocytes • integrins • angina

Adhesion of neutrophils and monocytes to endothelial cells is the initial event in the acute inflammatory response and in the pathogenesis of vascular disease.[1-3] Recent experimental and clinical observations have begun to define the molecular determinants on the surface of leukocytes that contribute to the adhesive process.[4-6] These studies have established the in vivo critical role of CD11/CD18 leukocyte adhesion molecules, a family of cell-surface glycoproteins consisting of three heterodimers sharing a common β-subunit with a distinct α-subunit (CD11a, CD11b, CD11c). Regarding adhesion to endothelial cells, CD11b/CD18 and CD11a/CD18 receptors are particularly important.[4] Whereas CD11a/CD18 is constitutively expressed in the plasma membrane and is not upregulated, CD11b/CD18 can be upregulated severalfold from intracellular granules by chemotactic factors such as C5a, interleukin-8, and platelet activating factor.[3-5]

Adhesion of neutrophils and monocytes to endothelial cells of coronary arteries and subsequent leukocyte activation may be relevant in the progression and evo-

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lution of atherosclerotic coronary disease.[7-11] Recent data also suggest a role for inflammation in the pathophysiology of unstable angina.[12-14] This study was undertaken to assess whether upmodulation of CD11b/CD18 adhesion receptors of neutrophils and monocytes occurs during the passage of blood through the coronary tree of patients with coronary heart disease.

Methods

Study Patients

Thirty-nine patients who underwent diagnostic coronary arteriography were studied. All patients gave writ-

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TABLE 1. Monocyte and Granulocyte Counts in Aorta and Coronary Sinus in the Three Groups of Patients

	Monocytes		Granulocytes	
	Aorta	Coronary sinus	Aorta	Coronary sinus
Group 1	240±0.06	280±0.04	3600±0.40	3800±0.80
Group 2	180±0.07	260±0.05	3400±0.60	3600±0.50
Group 3	240±0.05	200±0.04	3900±0.80	3800±0.70

Values are mean±SEM (mm^3).

ten informed consent, and the protocol was approved by the hospital ethics committee. Fifteen patients (group 1) had a clinical diagnosis of unstable angina defined as class IIB or IIIB of the Braunwald classification. All these patients had chest pain at rest associated with transient ST-segment changes on ECG unaccompanied by serum creatine kinase elevation or a new Q wave in the ECG.[14] Fourteen patients (group 2) suffered from stable exertional angina and had a positive exercise test defined as the development of ST-segment depression of >1 mm. The remaining 10 patients (group 3) complained of chest pain but had a negative exercise test. Cardioactive drugs, which included nitrates, β-blockers, and calcium antagonists, were not discontinued in most of the patients at the time of coronary arteriography. Group 1 patients were also taking antiaggregating agents (12 patients, aspirin; 3 patients, ticlodipine). At the time of the study, no patient had congestive heart failure or an acute infective disease. No attempt was made to alter the patients' medications, and those taking nonsteroidal anti-inflammatory drugs or steroids were excluded. Significant coronary artery disease was defined as >50% narrowing in the luminal diameter of any individual coronary vessel.

Study Protocol

Patients were studied in the fasting state after premedication with diazepam (10 mg). At the beginning of the procedure, an 8F pigtail catheter was advanced from the femoral artery and placed in the ascending aorta, and a 7F Gorlin catheter was inserted in an antecubital vein and placed in the coronary sinus. Simultaneous sampling (5 mL of heparinized blood) was obtained from the aorta and coronary sinus before the injection of contrast medium. Aortic and coronary sinus blood was collected into heparinized plastic syringes and immediately placed in polystyrene tubes at 4°C.

Monoclonal Antibodies and Direct Immunofluorescence

Cell surface receptors were detected by direct immunofluorescence evaluated by flow cytometry. The leukocytes were stained in whole (unseparated) blood to avoid any in vitro manipulation that might activate phagocytes.[15] The following monoclonal antibodies were tested: OKM1 (Ortho Diagnostics, Milan, Italy), used as the anti-CD11b/CD18 complex; LFA-1 (Sorin Biomedica Saluggia, Italy), used against the CD11a/CD18 complex; T11-FITC (Coulter Immunology, San Giuliano Milanese, Italy), the MoAb against the T-lymphocyte CD2 receptor; and Mo2-FITC (Coulter Immunology, San Giuliano Milanese, Italy), used against the monocyte CD14 receptor. Anti-mouse control FITC was obtained from AMD Alma Export, Firenze, Italy. All these MoAbs were incubated at a final concentration of 10 μL per 100 μL of whole blood for 30 minutes on ice, washed twice before and after lysis of erythrocytes by adding 2 mL of ice-cold erythrocyte-lysing solution (NH_4Cl 2.08 g; Na_2EDTA, 0.0108 g; $NaHCO_3$ 0.21 g in 250 mL H_2O), and analyzed. Nonspecific immunofluorescence was determined by using the control MoAbs, and the cell purity for granulocytes and monocytes was evaluated on the bitmap gating of the flow cytometer. Analysis was performed by flow cytometry using a fluorescence-activated cell sorter (Epics Profile II, Coulter Immunology). The laser 488-nm band was run at 500 mW of power. Gating for granulocytes and monocytes was determined by the dot blot generated by forward angle versus right angle scatter. Fluorescence and forward angle scatter of microsphere DNA check (Coulter Immunology) were used for instrument calibration. Fluorescence intensity of each cell was recorded as a mean channel number over the logarithmic range of 1 to 1024. The quantity of mean per cell expression of membrane glycoproteins was reported as mean channel of fluorescence intensity (Log/FL). Statistical analysis was performed with the Student's *t* test for paired data calculated with the Apple IIsi STATVIEW program.

Results

Clinical and Angiographic Data

In group 1, there were 11 men and 4 women, with a mean age of 54 years (range, 41 to 69). Ten patients had single-vessel disease and five had multivessel disease; mean ejection fraction was .59 (range, .46 to .80). In group 2, there were 13 men and 1 woman, with a mean age of 58 years (range, 48 to 70). Six patients had single-vessel disease and eight had multivessel disease, with a mean ejection fraction of .52 (range, .24 to .76). Group 3 included seven men and three women, with a mean age of 56 years (range, 35 to 70). All patients had normal coronary arteries: Three had mild to moderate mitral insufficiency caused by mitral valve prolapse and three had mild to moderate aortic regurgitation. Mean ejection fraction was .60 (range, .54 to .74).

Expression of Neutrophil and Monocyte Adhesion Receptors

Mean neutrophil and monocyte counts are shown in Table 1: No difference was found within each group between coronary sinus and aortic blood. Percentage of positive cells is shown in Table 2. More than 90% of monocytes and granulocytes bound LFA-1 and OKM1 (the MoAbs against the CD11a/CD18 and the CD11b/CD18 complex). The T-lymphocyte-specific anti-CD2 was bound by only 1% or less of cells, whereas the monocyte-specific anti-CD14 was bound by 94% to 99% of monocytes and by less than 1% of granulocytes, indicating the purity of the bitmap gating for granulocytes and monocytes analyzed by the flow cytometer.

TABLE 2 Percentage of Positive Cells on Bitmap Gating of Monocytes and Granulocytes in Aorta and Coronary Sinus in the Three Groups of Patients

	Monocyte bitmap (% of positive cells)						Granulocyte bitmap (% of positive cells)					
	Aorta			Coronary sinus			Aorta			Coronary sinus		
MoAbs	G1	G2	G3	G1	G2	G3	G1	G2	G3	G1	G2	G3
Negative control	0.1	0.2	0.4	0.3	0.4	0.2	0.8	0.9	0.9	0.9	0.8	1.0
Anti-CD2	0.8	0.7	1.0	1.0	0.6	0.6	0.9	0.5	0.9	0.9	0.8	0.5
Anti-CD14	96.0	94.3	99.0	96.0	97.0	97.9	0.8	0.6	1.4	1.2	0.9	0.8
Anti-CD11a/CD18	94.5	99.0	98.7	98.0	92.6	93.4	98.5	99.0	98.7	99.0	99.6	98.4
Anti-CD11b/CD18	97.4	98.4	99.1	98.3	97.6	99.2	98.6	98.4	98.1	99.0	99.0	98.4

G1, group 1 (unstable angina); G2, group 2 (stable angina); G3, group 3 (normal coronary arteries).

Table 3 shows the individual values of the mean channel of the fluorescence intensity for the anti-CD11b/CD18 complex of granulocytes and monocytes. In patients with unstable angina, neutrophils and monocytes showed a significantly higher expression of the CD11b/CD18 complex in the coronary sinus than in aortic blood ($P<.01$ and $P<.01$, respectively; Figs 1 and 2). Such difference was not observed in patients with stable angina or in those with normal coronary arteries. No difference was found in CD11a/CD18 expression from monocytes and granulocytes between coronary sinus and aortic mean channel values in each of the three patient groups (Fig 3).

Discussion

In this study, neutrophils and monocytes taken from the coronary sinus of patients with unstable angina were found to have increased expression of CD11b/CD18 adhesion receptors, thus demonstrating that an inflammatory reaction takes place in the coronary tree of patients with unstable angina. In vitro and in vivo studies show that activation of neutrophils results in upregulation of these surface glycoproteins. The chemotactic peptide fMLP is able to induce a threefold increase in CD11b expression on purified neutrophils pretreated with cytochalasin-B and with platelet activating factor.[15] The importance of the CD11/CD18 integrin for tissue injury in vivo has been demonstrated in a number of animal models. Addition of anti-CD18 MoAbs can reduce tissue injury and mortality in ischemia reperfusion-induced shock in rabbits[16] and myocardial infarct size in dogs.[17] Moreover, neutrophils accumulating at sites of acute inflammation in vivo have been shown to increase their surface density of CD11b/CD18 receptors compared with intravascular neutrophils simultaneously isolated from the same animals.[18,19] The physiological significance of enhanced neutrophil CD11b/CD18 expression[20] is also underlined by its detection in clinically active systemic lupus erythematosus[21] as well as in the synovial fluid of patients with rheumatoid arthritis.[22,23]

In our study, upregulation of the CD11b/CD18 complex was not accompanied by any difference between aortic and coronary sinus leukocyte count. This finding seems to exclude entrapment of these cells in the coronary vasculature. Adhesion of leukocytes to endothelial cells is a complex phenomenon involving receptors and ligands on both surfaces, and it is possible that stimulation of CD11b/CD18 receptor expression was insufficient to promote trapping of neutrophils in microvessels. In this study we did not correlate our findings with other biochemical indices of neutrophil activation such as elastase release. However, Dinerman et al[13] recently found that plasma levels of a neutrophil elastase-derived fibrinopeptide (B-β 30-43) were 13-fold higher in patients with unstable angina compared with control subjects. Previous data had suggested an inflammatory component in "active" angina. Berk et al[12] observed high levels of C-reactive protein, an interleukin-1 acute-phase reactant, in the peripheral blood of patients with unstable angina. Furthermore, adventitial infiltration of inflammatory cells involving (in most cases) autonomic nerve fibers was observed at autopsy in 12 patients with crescendo angina at rest.[24] In nine of such cases, a coronary thrombus superimposed on atherosclerotic coronary plaques was also found. Moreover, Forman et al[25] reported the case of a patient with variant angina complicated by sudden death in whom mast cell infiltration was found at the site of angiographic documentation of coronary spasm. These pathological observations provide a link between an active atherosclerotic process and an inflammatory response in patients with unstable angina culminating in sudden cardiac death.

Chemotactic factors able to induce activation of neutrophils and monocytes are potentially released into the coronary circulation of patients with unstable angina. The presence of an acute thrombotic process leads to increased plasmin activity, which can cause the activation of the complement system. Yasuda et al[26] found increased plasma levels of iC3b and C3d in 17 patients with unstable angina, suggesting complement activation in this clinical setting. Moreover, the terminal C5b-9 has been localized in atherosclerotic plaques and could come in contact with the circulating blood after plaque rupture.[27] Likewise, break or ulceration of coronary lesions may provoke the release of tumor necrosis factor (TNF)-α, which is a constituent of coronary atherosclerotic plaques.[23,28] This cytokine can induce neutrophil-mediated endothelial damage,[29] and recent studies suggest that a specific CD11b/CD18-mediated signal triggers toxicity of TNF-activated neutrophils.[28-30]

Activation of neutrophils and monocytes may have important consequences in unstable angina because these leukocytes, once stimulated, may release a variety of potentially toxic and vasoactive substances, in partic-

TABLE 3. Percentage Positivity of Granulocytes and Mean Channel of Fluorescence Intensity for the Anti-CD11b/CD18 Complex

	Monocytes				Granulocytes			
	Aorta		Coronary sinus		Aorta		Coronary sinus	
Patients	%	MC	%	MC	%	MC	%	MC
Group 1								
1	98.7	33.8	100.0	49.5	99.0	21.4	98.9	29.2
2	94.6	26.6	99.7	44.5	98.7	7.4	97.8	8.4
3	98.7	8.9	99.4	33.8	99.4	5.5	97.9	66.5
4	95.4	5.9	98.7	35.9	98.6	10.8	99.5	14.8
5	98.7	11.3	91.7	14.3	97.4	5.9	99.7	22.5
6	94.6	5.7	92.8	28.6	94.8	2.5	98.9	15.7
7	99.0	7.6	98.4	36.6	98.7	3.7	99.9	14.0
8	98.9	9.0	99.6	52.4	100.0	7.1	100.0	21.4
9	96.5	12.3	99.7	23.5	98.4	4.4	98.6	28.7
10	99.0	11.4	98.4	11.3	99.2	10.6	99.9	14.2
11	100.0	20.8	99.0	28.5	98.2	5.9	97.4	8.3
12	98.8	12.6	98.6	23.5	99.1	2.5	99.8	7.5
13	94.8	14.5	99.6	24.6	99.4	15.9	98.4	118.5
14	98.7	9.5	100.0	11.8	100.0	4.3	99.9	5.0
15	94.8	9.5	99.4	17.6	99.0	9.4	97.8	28.7
Total±ES	97.4±0.5	13±2.0	98.3±0.6	29.9±3.3	98.6±0.6	7.8±1.3	98.0±0.2	26.8±3.6
Group 2								
1	99.0	33.9	98.9	38.6	99.0	10.8	98.6	9.6
2	98.7	35.9	100.0	32.7	98.7	3.7	99.6	4.2
3	99.6	9.1	98.6	12.2	99.6	10.8	100.0	11.2
4	96.5	6.6	98.7	16.5	96.8	6.9	98.6	7.4
5	95.4	5.8	99.0	6.7	99.4	3.0	98.4	13.2
6	98.4	9.7	98.0	5.8	98.6	4.4	97.6	4.8
7	97.6	2.4	92.4	9.8	99.7	7.7	98.7	5.6
8	98.7	4.6	94.7	3.6	98.7	4.9	99.6	4.8
9	98.6	9.8	99.0	5.7	97.4	6.3	100.0	5.9
10	99.4	7.6	98.0	6.7	98.6	6.9	99.7	10.6
11	98.6	8.4	99.0	8.4	99.4	7.0	99.7	8.7
12	99.5	10.2	96.0	9.7	100.0	6.9	98.4	8.7
13	98.6	11.8	98.6	10.2	97.6	6.9	98.4	8.7
14	100.0	9.4	96.8	9.4	94.7	7.3	99.5	8.9
Total±ES	98.4±0.3	11.8±2.6	97.6±0.5	12.5±2.5	98.4±0.3	6.53±0.6	98.0±0.2	8.02±0.72
Group 3								
1	99.4	21.7	99.4	22.8	98.9	11.4	99.5	10.8
2	98.7	7.0	99.5	6.8	98.9	14.5	99.4	15.2
3	99.6	5.3	100.0	5.7	95.6	5.1	97.3	8.4
4	98.7	6.7	99.6	8.4	97.8	5.2	99.3	11.0
5	99.6	8.4	100.0	7.5	94.4	8.4	98.3	3.8
6	100.0	6.5	98.6	5.5	98.3	11.8	96.3	7.5
7	98.6	7.6	99.4	5.6	98.4	6.4	98.4	5.4
8	99.4	5.8	98.2	7.2	99.1	8.7	99.6	7.5
9	97.6	5.6	99.6	7.6	100.0	8.5	98.7	4.8
10	100.0	9.2	98.6	7.6	100.0	8.3	99.0	7.9
Total±ES	99.1±0.2	8.38±1.5	99.2±0.2	8.56±1.6	98.1±0.5	8.8±0.9	98.4±0.3	8.2±1.0

MC, mean channel values.

ular, the lipoxygenase-derived metabolites of arachidonic acid, leukotrienes C4, D4, and E4.[9,31] These substances have been shown to induce coronary vasoconstriction and decrease coronary flow in a variety of preparations. In addition, the respiratory burst results in the formation of oxygen-derived free radicals, which are able to alter microvascular permeability and influence vascular smooth muscle tone. Moreover, activated monocytes release interleukin-1, which induces biosynthesis and cell surface expression of procoagulant activity in cultured endothelial cells.[32] Recently, Neri Serneri et al[33] found that monocytes from patients with

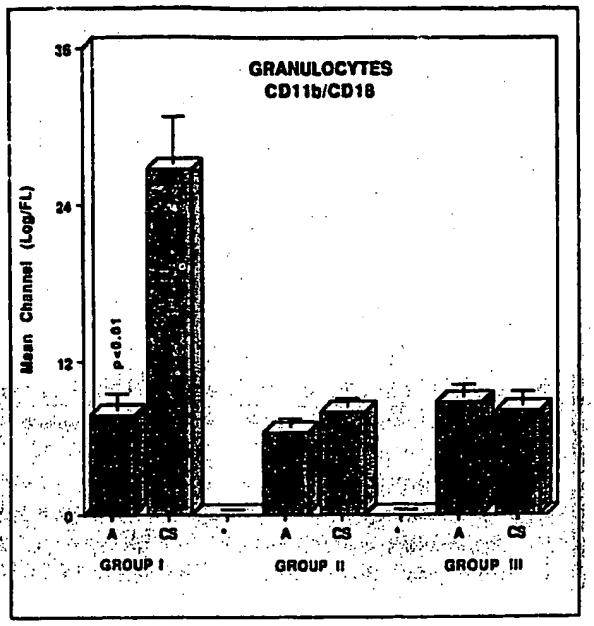

FIG 1. *Graph: Granulocytes from coronary sinus (CS) blood samples of group I patients (unstable angina) had a significantly higher expression of CD11b/CD18 adhesion receptor than granulocytes from aortic blood (A). Results are shown as mean channel of fluorescence intensity (Log/FL). No difference was found in the other group of patients.*

unstable angina showed high tissue factor–like procoagulant activity, whereas preparations from patients with stable angina and normal control subjects developed only small amounts of procoagulant activity. These authors speculated that activation of monocytes may be responsible for the increased thrombin formation in unstable angina. Finally, leukocyte-derived products

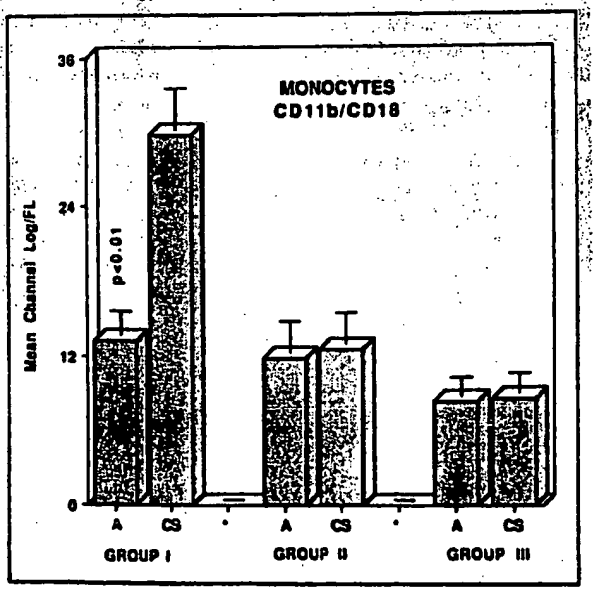

FIG 2. *Graph: Monocytes from coronary sinus (CS) blood samples of group I (unstable angina) showed a significantly higher expression of the CD11b/CD18 adhesion receptor than monocytes from aortic blood (A). Results are shown as mean channel of fluorescence intensity (Log/FL). No difference was seen in the other two groups.*

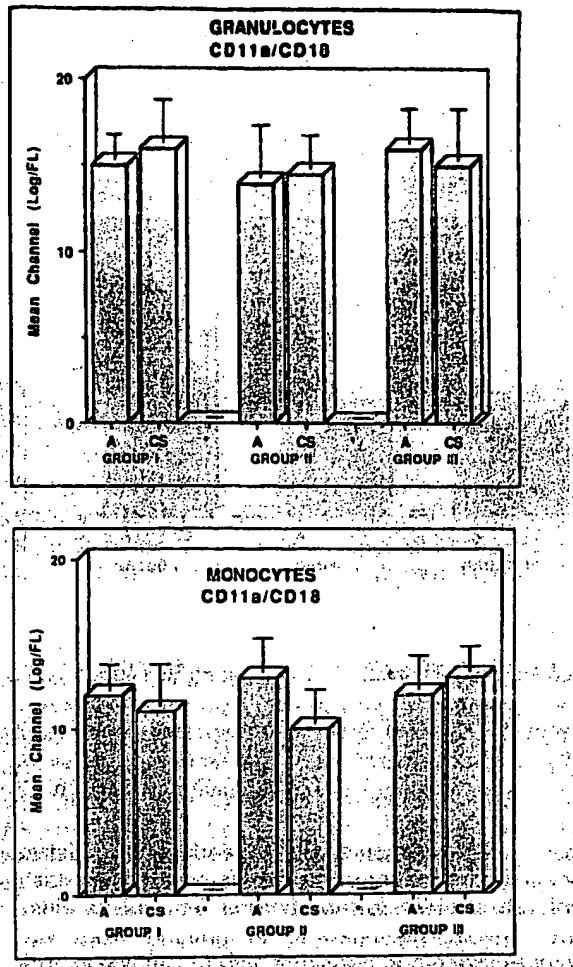

FIG 3. *Graphs: Expression of the CD11a/CD18 complex of granulocytes and monocytes in coronary sinus (CS) and aortic (A) blood samples in the three groups of patients. Results are shown as mean channel of fluorescence intensity (Log/FL).*

enhance platelet aggregation, whereas products of platelet activation may aid neutrophil accumulation at inflammatory sites.[34-36] Therefore, in unstable angina, activated leukocytes and platelets potentiate each other's effects, favoring the occurrence of coronary vasoconstriction and thrombosis.

References

1. Malech HL, Gallin JI. Neutrophils in human diseases. *N Engl J Med.* 1987;317:687-694.
2. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med.* 1989; 320:365-377.
3. Toothill VJ, Van Mourik JA, Niewenhuis HK, Metzelaar MJ, Pearson JD. Characterization of the enhanced adhesion of neutrophil leukocytes to thrombin-stimulated endothelial cells. *J Immunol.* 1990;145:283-291.
4. Smith CW, Rothlein R, Hughes BJ, Mariscalco MM, Rudloff HE, Schmalstieg FC, Anderson DC. Recognition of an endothelial determinant for CD18-dependent human neutrophil adherence and transendothelial migration. *J Clin Invest.* 1988;82:1746-1756.
5. Detmers PA, Wright SD. Adhesion-promoting receptors on leukocytes. *Curr Opin Immunol.* 1988;1:10-15.
6. Harlan JM. Leukocyte-endothelial cell interactions. *Schweiz Med Wochenschr.* 1991;121(suppl 43):7-10.
7. Mehta J, Dinerman J, Mehta P, Saldeen TG, Lawson D, Donnelly WH, Wallin R. Neutrophil function in ischemic heart disease. *Circulation.* 1989;79:549-556.

8. Ricevuti G, De Servi S, Mazzone A, Angoli L, Ghio S, Specchia G. Increased neutrophil aggregability in coronary artery disease. *Eur Heart J.* 1990;11:814-819.
9. De Servi S, Ricevuti G, Mazzone A, Pasotti D, Bramucci E, Angoli L, Specchia G. Transcardiac release of leukotriene C_4 by neutrophils in patients with coronary artery disease. *J Am Coll Cardiol.* 1991;17:1125-1128.
10. Ricevuti G, Mazzone A, Pasotti D, De Servi S, Specchia G. Role of granulocytes in endothelial injury in coronary heart disease in humans. *Atherosclerosis.* 1991;91:1-14.
11. De Servi S, Ricevuti G, Mazzone A, Ghio S, Zito A, Raffaghello S, Specchia G. Granulocyte function in coronary artery disease. *Am J Cardiol.* 1991;68:64B-68B.
12. Berk BC, Weintraub WS, Alexander RW. Elevation of C-reactive protein in "active" coronary artery disease. *Am J Cardiol.* 1990;65:168-172.
13. Diherman JL, Mehta JL, Saldeen TGP, Emerson S, Wallin R, Davda R, Davidson A. Increased neutrophil elastase release in unstable angina pectoris and acute myocardial infarction. *J Am Coll Cardiol.* 1990;15:1559-1563.
14. Ardissino D, Barberis P, De Servi S, Merlini PA, Bramucci E, Falcone C, Specchia G. Abnormal coronary vasoconstriction as a predictor of restenosis after successful coronary angioplasty in patients with unstable angina pectoris. *N Engl J Med.* 1991;325:1053-1057.
15. Kuijpers TW, Tool ATJ, van der Schoot, Ginset LA, Onderwater JJM, Roos D, Verhoeven AJ. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood.* 1991;78:1105-1111.
16. Vedder NB, Winn RK, Rice CL, Chi EY, Arfors KE, Harlan JM. A monoclonal antibody to the adherence-promoting leukocyte glycoprotein, CD18, reduces organ injury and improves survival from hemorrhagic shock and resuscitation in rabbits. *J Clin Invest.* 1988;81:939-947.
17. Simpson PJ, Todd RF III, Fantone JC, Mickelson JK, Griffin JD, Lucchesi BR. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti CD11b) that inhibits leukocyte adhesion. *J Clin Invest.* 1988;81:624-633.
18. Rot A. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol Today.* 1992;13:291-294.
19. Freyer DR, Morganroth ML, Todd RF III. Surface Mo1 (CD11b/CD18) glycoprotein is up-modulated by neutrophils recruited to sites of inflammation in vivo. *Inflammation.* 1989;13:495-505.
20. Berger M, O'Shea J, Cross AS, Folks TM, Chused TM, Brown EJ, Frank MM. Human neutrophils increase expression of C3bi as well as C3b receptors upon activation. *J Clin Invest.* 1984;74:1566-1571.
21. Buyon JP, Shadick N, Berkman R, Hopkins P, Dalton J, Weissman G, Winchester R, Abramson SB. Surface expression of Gp 165/95, the complement receptor CR3, as a marker of disease activity in systemic lupus erythematosus. *Clin Immunol Immunopathol.* 1988;46:141-149.
22. Emery P, Lopez AF, Gordon GF, Vadas MA. Synovial fluid neutrophils of patients with rheumatoid arthritis have membrane antigen changes that reflect activation. *Ann Rheum Dis.* 1988;47:34-39.
23. Yuo A, Kitagawa S, Kasahara T, Matsushima K, Saito M, Takaku F. Stimulation and priming of human neutrophils by interleukin-8: cooperation with tumor necrosis factor and colony-stimulating factors. *Blood.* 1991;78:2708-2714.
24. Kohci K, Takebayashi S, Hiroki T, Nobuyoshi M. Significance of adventitial inflammation of the coronary artery in patients with unstable angina: results at autopsy. *Circulation.* 1985;71:709-716.
25. Forman MB, Oates JA, Robertson D, Robertson RM, Roberst LJ, Virmani R. Increased adventitial mast cells in a patient with coronary spasm. *N Engl J Med.* 1985;313:1138-1141.
26. Yasuda M, Takeuchi K, Hiruma M, Iida H, Tahara A, Itagane H, Toda I, Akioka K, Teragaki M, Oku H. The complement system in ischemic heart disease. *Circulation.* 1990;81:156-163.
27. Niculescu F, Rus HG, Vlaicu R. Activation of the human terminal complement pathway in atherosclerosis. *Clin Immunol Immunopathol.* 1987;45:147-155.
28. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS. Detection and localization of tumor necrosis factor in human atheroma. *Am J Cardiol.* 1990;65:297-302.
29. Von Asmuth EJU, Van Der Linden CJ, Leeuwenberg JFT, Buurman WA. Involvement of the CD11b/CD18 integrin but not of the endothelial cell adhesion molecules ELAM-1 and ICAM-1 in tumor necrosis factor-α-induced neutrophil toxicity. *J Immunol.* 1991;142:3869-3875.
30. Nathan C, Srimal S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright SD. Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J Cell Biol.* 1989;109:1341-1352.
31. Feurstein G, Hallenbeck JM. Leukotrienes in health and disease. *FASEB J.* 1987;1:186-192.
32. Harlan JM. Leukocyte-endothelial interactions. *Blood.* 1985;65:513-525.
33. Neri Serneri GG, Abbate R, Gori AM, Attanasio M, Martini F, Giusti B, Dabizzi P, Poggesi L, Modesti PA, Trotta F, Rostagno C, Boddi M, Gensini GF. Transient intermittent lymphocyte activation is responsible for the instability of angina. *Circulation.* 1992;86:790-797.
34. Del Maschio A, Evangelista V, Rajtar G, Chen ZM, Cerletti C, De Gaetano G. Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am J Physiol.* 1990;258:H870-H879.
35. Bazzoni G, Dejana E, Del Maschio A. Platelet-neutrophil interactions: possible relevance in the pathogenesis of thrombosis and inflammation. *Haematologica.* 1991;76:491-499.
36. Faint RW. Platelet-neutrophil interactions: their significance. *Blood Rev.* 1992;6:83-91.

Novel and Emerging Therapies in Cardiology and Haematology

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Abstract: Reviewing advances in cardiology and haematology together may appear at first sight to require some artificiality to make a satisfying fit. For two reasons, at least, this is not the case. Firstly, convergence in biology has become very clear over the past decade and this could not be better illustrated by the demonstration that the haemangioblast is the common progenitor of both haemapoietic stem cells and vascular endothelium. This opens the way to common (and differential) approaches to the manipulation of these cells, a field at present in its infancy. A second convergence is the common goal of understanding the processes resulting in haemostasis, thrombosis and vascular occlusion and the means for developing effective antithrombotics. This is exemplified by a number of agents either in use or in clinical trial as a result of haematological and cardiological collaboration. This collaboration is recognisable with the development, many years ago, of streptokinase and the use of aspirin in vascular disease and continues to this day with specific antiplatelet inhibitors and oral thrombin inhibitors as they become accepted into clinical use over the next few years. Here we review current advances in pharmacological treatments in cardiology and haematology, grouped primarily by disease process, focusing on novel and emerging therapies likely to be of importance in the future.

INTRODUCTION

Despite significant advances in pharmacologic and non-pharmacologic therapies, cardiovascular disease remains the leading cause of morbidity and mortality in the industrialized world. Pharmacologic approaches have been the mainstay of treatment for many cardiovascular disorders, including acute coronary syndromes, chronic coronary artery disease, hypertension, heart failure, and dysplipidemias. Presently however, the greatest area of change within cardiology is in interventional cardiology, where percutaneous revascularisation techniques such as angioplasty and stenting are being increasingly used in the acute and chronic management of coronary and peripheral vascular disease. Advances in these revascularisation techniques are dependent on improved adjunctive agents, principally anti-platelet, anti-thrombotic and anti-restenotic drugs, such that pharmacologic therapies at present are key to the overall treatment of most cardiovascular disorders.

The major therapeutic advances in haematology have historically been sporadic rather than sustained and have often occurred in tandem with seminal discoveries in the fields of physics, biochemistry and cell biology. Following years of experimenting with different combinations of the same drugs the spectacular advances in molecular medicine and cell biology in the last two decades have provided the main impetus to the design of novel therapies in haematology in recent years. These advances have been applied primarily to the malignant hematological disorders, which have traditionally provided insights into gene regulation and cell cycle control but have been difficult to cure. Although these advances have led more to a realization of the enormous complexity of the field than a plethora of novel therapeutic applications, they provide a platform for future drug design and are worthy of study.

This review discusses current advances in pharmacological treatments in cardiology and haematology, grouped primarily by disease process, and focusing on those novel and emerging therapies likely to play important future roles.

NOVEL AND EMERGING THERAPIES IN INTERVENTIONAL CARDIOLOGY

Interventional cardiology refers to the percutaneous treatment of coronary artery disease, by means of mechanical dilation of obstructive coronary lesions. Percutaneous treatments have included coronary angioplasty, or balloon dilation of obstructive lesions; but now also increasingly include the use of coronary stents – metal mesh sleeves deployed into the vessel wall by balloon inflation – to achieve optimum results [1]. The success of these procedures has been limited in the long term by progressive vessel renarrowing or restenosis; and in the short term by platelet initiated thrombosis at the site of dilation or stent deployment. Significant recent pharmacologic advances have been made in both of these areas.

Strategies to Reduce Restenosis

Restenosis, the "arterial healing response following injury incurred during transluminal coronary

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revascularisation" [2], has long been the principal drawback of coronary intervention. Although stents are associated with restenosis rates of 20-30%, compared to 30-40% for balloon angioplasty alone in randomised trials of certain patient subsets [3], restenosis rates remain high in other populations, such as those with diabetes, or complex lesions, such that in-stent restenosis (ISR) has itself become an important problem [3]. From the 1980's to the present, numerous drugs tested in animal models – particularly the pig - have suggested benefit [2,4], although until recently none have shown benefit in humans [3]. Therapies in 3 broad areas have recently emerged.

Gene Transfer

Gene transfer involves the direct introduction of a given gene into a local environment, in order to increase the expression and function of that gene to gain pathophysiologic benefit [5]. Restenosis has in many ways been seen as an ideal pathogenic process to be treated by gene transfer, in that the onset of the restenotic process is largely known and the site of restenosis is localised and readily accessible [6], both for percutaneous catheter-based delivery methods, and for surgical approaches. In addition, the restenotic process is itself relatively short-lived, largely complete within 3-6 months [3], and the internal and external elastic laminae themselves provide physical barriers such that transfected gene expression can be localised to specific layers [7]

In general, gene transfer can be achieved by transfection, direct physical or chemical transfer; or by transduction, gene transfer using viral vectors [8]. Direct physical transfer of naked plasmid DNA has been demonstrated by injection into myocardium, both by open surgical techniques [9] and using catheters placed in the ventricular cavity [10]. A drawback with this technique is low efficiency: only a small proportion of delivered DNA reaches the target cells, and even less reaches the cell nucleus. Nevertheless, cells disrupted by mechanical injury may have increased uptake, and a number of studies have shown meaningful biologic effect even with low transfection efficiency [11].

Specifically in the setting of restenosis, naked plasmid DNA encoding for the 165 amino acid version of Vascular Endothelial Growth Factor (VEGF), delivered to the rabbit iliac artery using a hydrogel balloon results in 60% inhibition of neointima (NI) formation following stenting [3]. More recently, naked DNA in a polylactic-polyglycolic acid coated stent has been successfully delivered with high efficiency to normal porcine coronary arteries [12], suggesting this technique may be useful in the setting of restenosis. Chemical, usually lipid, solutions are an alternative. Large, branching lipid molecules form lipophilic coatings, surrounding DNA plasmids [13] and fuse with cell membranes, allowing cell delivery. This technique has been used for genes targeting Prostaglandin I2 (PGI2) in the rat and cecropin in the pig following angioplasty, with >80% inhibition of NI formation in each case [3].

There is more experience with viral vectors, and for adenoviruses in particular. Adenoviruses are double-stranded DNA viruses which can produce large amounts of purified recombinant virus, allowing efficient gene expression [14], which remains distinct from the host genetic material. However, adenoviruses may be associated with immune and inflammatory responses, particularly when given via the intraluminal route, and thrombus formation [15]. Transgene expression may also be transient. There is also experience with viral/lipid combinations, such as the haemagglutinating virus of Japan / liposome combination which has been used to target genes for p53 and eNOS in rat models of balloon angioplasty [3]. Retroviruses have also been used, though concerns remain as to the possibility of mutations at the sites of gene insertion [16].

Despite the localized nature of the restenotic process, delivery methods for gene transfer have been problematic. Initially open surgical approaches were used to isolate specific vessel segments; then catheter-based techniques aimed to achieve this same end, initially described in 1989 [17]. Since then, a wide variety of local delivery catheters have been described [18], aiming to provide selective local delivery while maintaining vessel perfusion. This large variety perhaps reflects the fact that none of these techniques is particularly efficient. Less than 1% of drug dose has been documented to achieve delivery to the vessel wall using porous balloons [19]. Delivery may also be highly variable, and may be into dissection planes and side branches [18].

A wide variety of gene products have been investigated as potential targets for gene transfer, and undergone initial evaluation in animal models and in human studies [3]. These genes have been selected to attempt modification of a number of processes, principally SMC proliferation, but also cell migation, thrombosis and endothelial function [20]. Inhibition of neointimal formation, generally in the order of 30-70% has been observed, and significantly, a number of these approaches have entered phase I human trials. Naked plasmid DNA and an adenovirus/lipid delivery combination are currently being used; plasmid DNA encoding for the 165 amino acid version of VEGF has been delivered in this way using a hydrogel and perfusion balloon approach, in human clinical trials for the prevention of peripheral arterial restenosis [11]. Early reports suggest this approach is well tolerated [21]. While studies of gene transfer for restenosis are still at an early stage, the results of preliminary human trials are promising. Important questions remain as to which genetic material should be targeted, which delivery method should be employed [22]. Concerns have also been raised over the possibility that increased growth factor activity may actually promote neointima and atheroma formation or plaque instability [21-2]. However, the potential advantages of using local gene delivery to treat a local iatrogenic process are significant, and likely to see continued research effort [6].

Gene Modification

Gene modification can be broadly defined as specifically targeting and inhibiting a regulatory gene that plays an important role in a pathogenic process, classically by creating a molecule of RNA or DNA that undergoes complementary base pairing with its endogenous target [23]. Gene modification to attempt reductions in restenosis has also been enthusiastically explored. Nucleic-acid based drugs can broadly be divided into four main types; antisense (AS), ribozymes (Rz's), DNAzymes (Dz's) and Decoys. AS molecules are single-stranded DNA molecules which form an RNA-DNA duplex with target mRNA [24]. AS oligodeoxynucleotides targeting the proto-oncogenes *c-myb*

and *c-myc* inhibit NI formation in rat and pig models of injury [25, 26], including stenting [27], although to date, the only reported use of AS to prevent ISR in humans using a locally-delivered AS molecule targeting *c-myc*, showed no benefit [28]. Rz's are RNA-based molecules with the advantage of the ability to cleave their target mRNA in an enzymatic fashion [29]. A Rz targeting *c-myb* inhibits SMC proliferation and NI formation after balloon injury to the rat carotid artery [30]. Similarly, Rz's targeting transforming growth factor-β_1 also inhibit NI formation in the rat model [31], and a chimeric DNA/RNA hammerhead Rz targeting proliferating cell nuclear antigen reduces NI formation by 28% in the pig coronary stent model [32].

A more recent refinement to nucleic acid gene targeting strategies has been the development of Dz's. These are single-stranded DNA molecules with catalytic domains capable of RNA cleavage at high efficiencies, with added stability conferred by their DNA structure and base modifications [33]. Locally-delivered Dz's targeting the zinc finger transcription factor early growth response factor-1 (Egr-1) inhibit NI formation in the rat carotid injury model [34], and more recently, Dz's targeting the human EGR-1 have been shown to inhibit ISR in the porcine coronary stent model to a similar degree [4]. These molecules are extremely versatile, and besides their obvious therapeutic potential, can be used as probes of gene structure and function in complex biological milieu.

A further approach is the use of decoys. These are double-standed oligonucleotides that interfere with transcription factor binding [35]. In the case of VSMC proliferation, a decoy targeting the transcription factor E2F is thought to act by preventing E2F binding to cyclinA/cdk 2 complex, normally key to the process of activation of a number of downstream genes involved in cell cycle regulation, including PCNA, c-myc and c-myb [36]. E2F decoys have reduced NI formation in the rat model of balloon angioplasty, and initial results from human clinical trials also suggest a reduction in vein graft occlusion rates.[37]

Drug-eluting Stents

The localised nature of restenosis, and the observation that most percutaneous coronary interventions now involve the use of a stent, has prompted efforts to use the stent itself as a drug-eluting device. Efforts to date have focused on the use of stents coated with anti-restenotic agents, but the principle of a drug-eluting stent may also apply in the localised treatment of acute coronary occlusion and thrombosis and even been extended to include the possibility of cell and gene delivery using a stent platform [38]. Polymer-coated stents initially provoked excessive reactions but subsequent coatings using phosphorylcholine and other polymers have not had this effect. Initial studies demonstrating heparin could be bonded to the stent struts - although subsequently with only limited use in clinical practice – initially demonstrated the principal of the drug-eluting stent [39].

Importantly, a number of agents have since been shown to elute slowly from polymer coatings and are associated with reduced NI formation in animal models. Two anti-proliferative agents - sirolimus (rapamycin) and paclitaxel, a taxol analogue - have undergone initial evaluation in human trials. Interestingly, both have proven efficacy in other antiproliferative contexts, sirolimus to reduce post transplant coronary neointimal hyperplasia, and taxol as an antineoplastic agent [39,40]. Sirolimus, is a naturally-occuring compound derived from the streptomyces fungus and stimulates p27kip1 levels causing cyclin-Cdk complex inhibition and cell cycle arrest. In 45 patients treated with a sirolimus-coated stent (140 μg/cm2), either as a fast (15 day) or slow (>28 day) release formulation, negligible NI regrowth has been observed at up to 12 months. Subsequently, 238 patients randomised to a sirolimus-coated stent or conventional stent for *de novo* coronary lesions, demonstrated a zero restenosis rate in the sirolimus group (v 26% $p<0.0001$), clearly a finding of huge implications [41]. This sirolimus-coated stent recently received approval from the Food and Drug Administration (FDA) in the US. Similarly, paclitaxel, a naturally occuring compound from the pacific yew tree with potent antiproliferative effects, thought to be due to an alteration in microtubular function, has shown promising early results in 32 patients stented with a paclitaxel derivative impregnated sleeve incorporated into a stent design [40]. Clearly there remain a number of questions with this approach, such as the effects of multiple or overlapping stents, the correct choice of agent, longer-term outcomes and toxicity, but the results of studies published to date have generated great interest.

Strategies to Reduce Thrombosis During Percutaneous Coronary Intervention

Percutaneous Coronary Interventions (PCIs) induce disruption of the endothelium, and exposure of the vessel wall to blood borne prothrombotic elements. This contributes to platelet activation, leading to varying degrees of platelet plug formation and arterial thrombosis, a tendency increased with the deployment of a prothrombotic stainless-steel stent. Thus the process of platelet activation and subsequent adhesion, aggregation, secretion and procoagulant activity has been the subject of intense research, to reduce the incidence of thrombosis following coronary intervention.

IIb/IIIa Receptor Antagonists

Congenital and acquired disorders of platelet function suggest that inhibition of any one of these steps in platelet function would be an effective means of platelet inhibitor therapy, although until recently drug development has focused in particular on the inhibition of the IIb/IIIa receptor [42]. Three types of IIb/IIIa receptor antagonists have been investigated to the extent of large phase III clinical trials. These are: the monoclonal Fab fragment termed abciximab; cyclic peptides based on the RGD (Arg-Gly-Asp) motif, and RGD-based pepidomimetics [42]. Examples of all 3 drug types have recently entered clinical use.

Abciximab is a recombinant protein, comprised of the Fab fragment of a chimeric human-murine monoclonal antibody 7E3, which targets the IIb/IIIa receptor on the platelet surface, and is the IIb/IIIa receptor antagonist for which there is the most clinical experience [42]. The EPIC trial published in 1994 demonstrated a 35% reduction in a composite endpoint of combined cardiovascular events with

the use of abciximab bolus and infusion compared to placebo in patients undergoing percutaneous coronary intervention (PCI), although this was associated with an increased risk of bleeding [43]. Subsequent trials confirmed efficacy and reduced morbidity with lower heparin doses [44] and following coronary stenting [45], and a benefit acutely, was observed in patients when abciximab was given in the setting of unstable angina pectoris (UAP) [46]. Abciximab also binds to the leucocyte integrin Mac-1 [47], and to the widespread integrin $\alpha_v\beta_3$ [48], although the physiologic significance of these interactions remains uncertain.

Eptifibatide is a synthetic cyclic heptapeptide based on the RGD motif that binds exclusively to the IIb/IIIa receptor, and has shown significant - though not profound - reduction in composite cardiovascular endpoints at 30 days following PCI compared to placebo [49]. Tirofiban is a synthetic RGD-based peptidomimetic tyrosine analogue. The results of clinical trials with this agent have been less impressive, with no sustained benefit seen following PCI, and a modest benefit observed when used in the setting of UAP and non-Q wave myocardial infarction (NQWMI) [50], for which it is approved clinically. In contrast to these intravenous agents, initially promising reports using orally active IIb/IIIa receptor antagonists have shown no benefit [51, 52].

ADP Receptor Antagonists

Two other commonly used anti-platelet agents are ticlopidine and clopidogrel, structurally very similar thienopyridines that impair ADP induced platelet aggregation, and commonly referred to as ADP receptor antagonists. Their precise mechanism of action is not known, but is thought to include: inhibition of ADP induced inhibition of adenylate cyclase and prevention of G protein association with the platelet membrane [53]. Both agents reduce cardiovascular events in patients with a history of vascular disease, reduce coronary stent thrombosis, but since clopidogrel has an improved side effect profile, use of this agent has become more widespread [53].

NOVEL AND EMERGING THERAPIES FOR ACUTE CORONARY SYNDROMES

Acute coronary syndromes include the clinical spectrum of UAP, NQWMI and Q wave myocardial infarction (MI). They share the pathogenic mechanism of rupture of the fibrous cap of an atherosclerotic coronary lesion, causing exposure of flowing blood to tissue factor and other elements, resulting in platelet activation, leading to platelet plug formation and arterial thrombosis in a manner analogous to that following PCI [54]. Likewise, the processes of platelet activation, adhesion, aggregation, secretion and procoagulant activity have also been a focus of research efforts to treat acute coronary syndromes.

Novel Antiplatelet Agents

In addition to aspirin and the antiplatelet agents discussed above, and given the multiple pathways of platelet activation, additional approaches to reduce platelet activation have been investigated. Many of these use receptor antagonists at a number of points within the cascade of activation {Fig. (1)} [55]. First, are approaches to inhibit vWF-GPIb interaction. Plasma von Willebrand factor (vWF) is a multimeric glycoprotein that mediates the adhesion of platelets to sites of vascular injury at arterial shear, and plays a critical role in the formation of an arterial thrombus. vWF, deposited onto subendothelial collagen [56]or other substrates [57] binds to the platelet membrane glycoprotein Ib receptor and tethers platelets in flowing blood to the vessel wall. Plasma vWF and fibrinogen, bind to adhered platelets via the platelet membrane integrin, $\alpha_{IIb}\beta_3$ and cross-link activated platelets at the site of vessel injury thereby promoting the progression of the platelet thrombus. GPIb bound plasma vWF appears to act in synergy with $\alpha_{IIb}\beta_3$ bound fibrinogen and vWF in sustaining platelet accrual [58]. Given the central role vWF plays in the initiation and progression of an arterial thrombus, interrupting the association between vWF and its ligands has long been considered a promising target for drug design but none are currently available.

Recombinant fragments of the vWF molecule that express the GPIb-binding region have been developed; 2 of these (Leu 504-Lys 728 and Ala 444-Asp 730) appear effective in animal models of thrombosis [59, 60]. Other approaches have been antibodies to GPIb which also appear effective in animal models of thrombosis, although given that these agents delay rather that eliminate thrombus formation, it is likely they will require adjunctive use of aspirin or other agents [55, 61]. A second approach is to inhibit platelet-collagen interaction. A number of naturally occurring compounds bind to collagen binding sites; rLAPP (recombinant leech antiplatelet protein) binds to the vWF binding domain, calin to the GPIa/IIa-I-binding site, both inducing an antithrombotic effect in mice [55]. Collagen recognizes 2 platelet receptors $\alpha_2\beta_1$ and GPVI; the identification of the peptide sequences in collagen that recognize these receptors and designing antagonists is therefore another approach, although $\alpha_2\beta_1$ is also present on a number of other tissues, which will likely reduce specificity of any agents developed. A third approach is to target thrombin-induced platelet activation. This normally occurs via 2 receptors; protease activated receptors PAR1 and PAR4. A variety of approaches have been employed to target these receptors; one such polyclonal antibody directed against the N-terminal exosite of cloned PAR1 has an effective antithrombotic action [62]. Another means of inhibiting thrombin-induced platelet activation is the naturally occurring compound thrombostatin [63]. Lastly, impairing ADP-platelet receptor P2Y1 and P2Y12 interactions can also impair platelet aggregation and thrombosis [55], and these agents are currently in phase II human clinical trials [64, 65].

Novel Anticoagulant Agents

Heparin and warfarin have been in use for over 50 years and formed the principal anticoagulants in clinical use until recently. Advances have been made in dosing and formulation of these agents, particularly the use of low molecular weight heparins (LMWH) in place of unfractionated heparin (UFH), with resultant greater ease of administration, predictability of effect, and less requirement for monitoring [66]. These improvements have been relatively modest compared to developments in the last

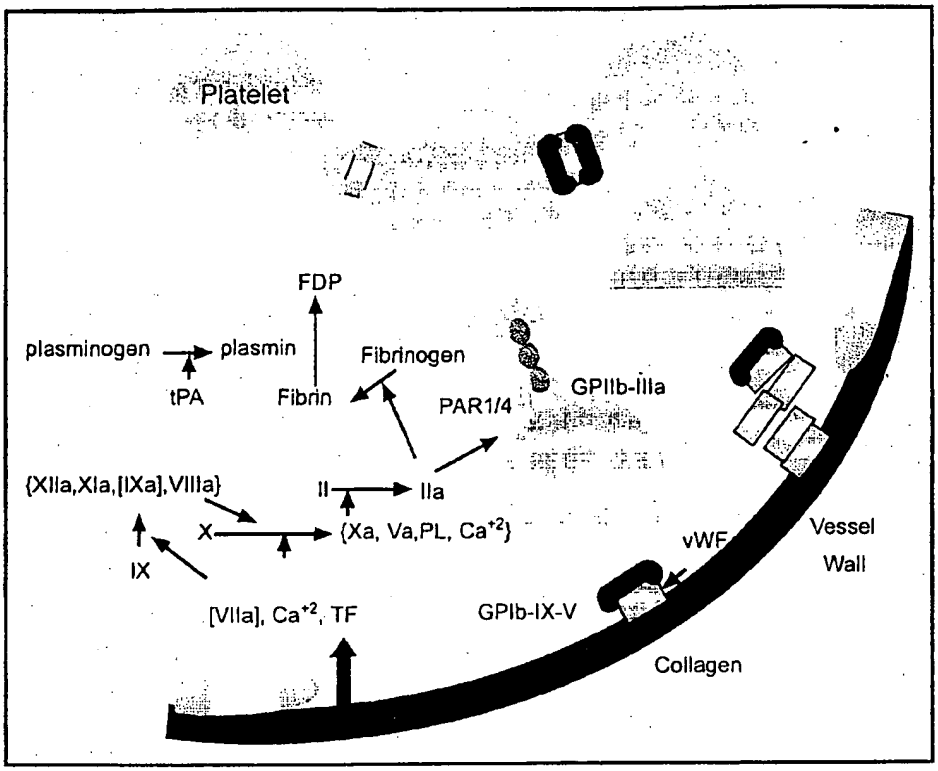

Fig. (1). The formation of a platelet fibrin thrombus. A schematic diagram representing the complex interaction between the subendothelium, platelets and the coagulation cascade. Under high shear, platelet adhesion is initiated through the binding of GP1bα to the A1 domain of subendothelial vWF. GPIb-vWF induced signaling leads to the activation of integrin $\alpha_{IIb}\beta_3$ and the firm adhesion of platelets once this receptor engages the RGD domain of vWF. Platelet GP1bα bound vWF can self associate with subendothelial vWF and also plays a role in platelet accrual along with integrin $\alpha_{IIb}\beta_3$ associated fibrinogen and vWF. The release of tissue factor from endothelial cells and adjacent fibroblasts promotes the formation of fibrin, a process that is regulated by specific inhibitors and also by fibrinolysis.

decade, in a multitude of novel proposed agents, targeting almost every step of the coagulation cascade in efforts to overcome some of the limitations of these drugs {Fig. (2)}. The coagulation cascade can be viewed in 3 stages; initiation, thrombin generation, and thrombin activation {Fig. (2)}. Novel specific inhibitors have been developed which act at each of these stages [66].

Initiation of coagulation occurs when free-flowing blood is exposed to tissue factor (TF). TF then binds factor VIIa to form TF/VIIa complex. Thrombin generation then occurs via activation of factors X and IX by the TF/VIIa complex. Activated IXa combines with VIIIa to convert X to Xa, which then combines with Va and thrombin (factor II). At present, a number of inhibitors of initiation of coagulation have been proposed. These include 2 naturally occurring compounds; Tissue factor pathway inhibitor (TFPI) [67] and nematode anticoagulant protein (NAPc2) [68]. NAPc2 inhibits Factor VIIa, but also binds Factors X and Xa, and has been evaluated for venous thrombosis prevention [66]. As shown in Fig. (2), there are multiple sites at which drugs may modify enzymatic steps in thrombin generation. The most experience has been gained with the Factor Xa inhibitors. These include Pentasaccharide (Org31540/SR90107A), antistatin and Tick Anticoagulant Peptide (TAP) [66]. Pentasaccharide (PS) is a synthetic molecule with high affinity for antithrombin (AT). PS also has the advantages that it does not activate platelets or bind to platelet factor 4, and does not contribute to heparin-induced thrombocytopaenia [69]. In 316 patients undergoing thrombolysis with r-tPA, treated with either PS or unfractionated heparin for acute MI, vessel patency rates were similar, though not improved, with PS. Further evaluation of this agent is underway [69].

Thrombin (factor II) is activated by Xa and Va. Direct thrombin inhibitors (DTIs) inactivate free and fibrin-bound thrombin. They are of two types: bivalent inhibitors (hirudin and bivalirudin) which act on 2 sites; the substrate recognition site and the active site; and the direct inhibitors which act only on the active site and include a number of smaller molecules (argatroban, efegatran, inogatran, melagatran and others). Hirudin is a synthetic 65 amino acid polypeptide originally derived from the leech salivary gland and bivalirudin is a 20 amino acid molecule, part of which is analogous to the carboxyterminal portion of hirudin.

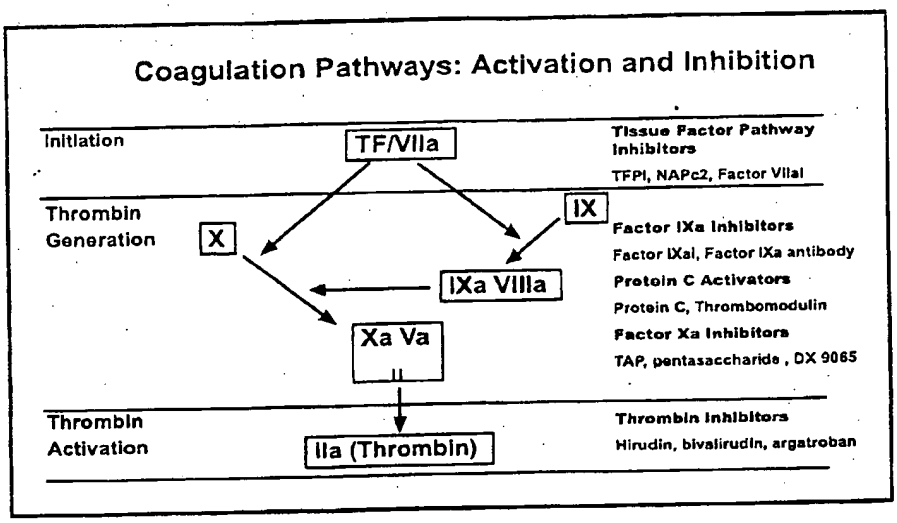

Fig. (2). The coagulation cascade. A simplified schematic outlining the coagulation cascade, with steps divided into initiation, thrombin generation and thrombin activation. The various classes of drug inhibitors of these steps are shown, and discussed in the text. TF = tissue factor. Adapted from Ref 191.

Hirudin had similar efficacy to heparin in reducing thrombotic complications in patients with UAP and NQWMI, although was associated with greater bleeding risk. Bivalirudin had a similar effect to heparin in patients undergoing PCI although it was associated with less bleeding [70]. In patients with acute MI, there is the early suggestion of improved vessel patency in combination with SK, and this approach is currently under trial in a large patient cohort. In contrast, the small molecule active site inhibitors have been disappointing for acute coronary syndromes, with no clear benefit over heparin demonstrated to date [66, 71]. At present, bivalirudin is the only DTI with FDA approval for use in acute coronary syndromes, as an alternative to heparin for patients undergoing percutaneous coronary intervention. Hirudin and argatroban have FDA approval only for patients in whom heparin is contraindicated because of heparin induced thrombocytopenia. The remaining DTIs are not approved for use [71].

Novel Antithrombotics

Although the role of vWF in atherogenesis and plaque instability is uncertain, it is of critical importance in the development of an occlusive thrombus following plaque rupture [72]; [73]. The atherosclerotic plaque is characterized by the presence of foamy macrophages and smooth muscle cells in the intimal layer of muscular arteries. It has been postulated that vWF, by promoting platelet adherence to the subendothelial matrix, facilitates the action of cytokines in promoting smooth muscle cell migration and proliferation [74]. Foamy macrophages are derived from circulating monocytes translocating to the subendothelium. The vWF propolypeptide, which is released along with mature vWF from endothelial cells, is a ligand for $\alpha_4\beta_1$, an integrin used by monocytes to adhere to endothelial cells. . It is possible that vWF participates in the recruitment of monocytes, either directly via the vWF propolypeptide or indirectly via platelets [75]; [76]. Mice can be made susceptible to atherosclerosis by breeding low density lipoprotein receptor (LDLR)-deficient mice and feeding them a diet rich in fat and cholesterol. Mice lacking both the vWF gene and the LDLR gene (LDLR $^{-/-}$: vWF $^{-/-}$) had 40% less atheroma at arterial bifurcation points than their counterparts with the vWF gene (LDLR $^{-/-}$: vWF $^{+/+}$) when fed a diet rich in fat and cholesterol [77]. A recent study using a rabbit model has demonstrated that vWF bound to endothelial cells helps recruit platelets to arterial branch points in response to hypercholesterolemia [78]. These studies support the view that vWF plays a significant role in atherogenesis at points of turbulent blood flow.

vWF circulates in blood as a series of multimers of varying size. Only the large and ultra large multimers are haemostatically competent. Large vWF multimers bind to activated platelets and collagen with up to ~100-fold higher affinity than monomeric fragments [79]. The larger the vWF multimer, the higher the number of binding sites and greater the potential for vWF-ligand interaction and formation of a competent platelet thrombus [80], [81], [82]. The role of vWF multimer size in atherogenesis is not known but the association between the size of the vWF multimer and its thrombotic potential is convincing and regulating vWF multimer size is an attractive option in the treatment of thrombotic disorders such as stroke, myocardial ischaemia and peripheral vascular disease.

vWF, is released from endothelial cells and platelets as ultra large multimers that are subsequently broken down into smaller and therefore less active forms by plasma factors. Two such factors, ADAMTS 13 and Thrombospondin-1 (TSP-1), have recently been identified. ADAMTS 13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif) reduces vWF multimer size by proteolytic cleavage of the vWF subunit. A deficiency of the vWF cleaving

protease and mutations in the ADAMTS 13 gene have been described in thrombotic thrombocytopaenic purpura (TTP), a microangiopathic haemolytic anaemia characterized by the presence of ultra large vWF multimers and diffuse arterial thrombosis [83-85]. TSP 1 is released from activated platelets and endothelial cells and regulates vWF multimer size, not by proteolytic cleavage but by reduction of the disulfide bonds, which link the individual subunits [86, 87]. We have recently localized the active site of TSP-1 to a free thiol at position 974 in the calcium-binding/C-terminal sequence [88]. The ability of these enzymes to regulate vWF multimer size, make them attractive options in the development of new antithrombotic drugs.

NOVEL TREATMENTS FOR ACUTE MYOCARDIAL INFARCTION

Thrombolytic Agents (Fig. (3))

Thrombolysis refers to the dissolution of thrombus, which has seen major application in the treatment of myocardial infarction, a process triggered by thrombotic occlusion of the epicardial coronary artery leading to downstream ischemia. Novel therapeutic thrombolytic agents have centred on either naturally occurring or synthetically produced enzymes.

The prototype thromboytic agent, Streptokinase (SK) was first derived from the beta haemolytic streptococcus. The name streptokinase is misleading; it is not an enzyme, but forms a 1:1 complex with plasminogen, which then causes conversion of plasminogen to plasmin. Plasmin then acts to degrade fibrin, promoting thrombus dissolution and improving arterial patency, alone and in combination with aspirin, above that of placebo; an effect translating into clinical benefit shown with SK in 1988 [89]. SK converts circulating as well as fibrin-bound plasminogen, whereas tissue type plasminogen activator (t-PA) a naturally occurring serine protease produced by the endothelium, acts solely on fibrin bound plasminogen activator, and is thought to induce earlier, higher rates of vessel patency than SK [90] in combination with heparin, particularly given as an accelerated dose [91]. This also translated into clinical improvements in mortality in a large clinical trial in 1993 [92]. However, given only 37% patency rates at 60 mins with t-PA translating to 30 day mortality rates generally staying above 7%, efforts have continued to select improved thrombolytic agents [93, 94]. These have been of a number of types, including mutant variations of t-PA, and other naturally occurring thrombolytics.

Mutant t-PAs include rPA, TNK and n-PA. r-PA (reteplase) is a deletion mutant of t-PA, consisting of just the kringle 2 and protease domains of the molecule, which binds more avidly to fibrin-bound plasmin and has a longer plasma half-life, allowing bolus delivery [95]. Despite promising initial studies, equivalent rather than improved, clinical outcomes have been demonstrated, compared to t-PA [94]. Similarly, TNK (TNK-t-PA or tenecteplase) is a mutant form of t-PA in which Thr 103 is substituted by

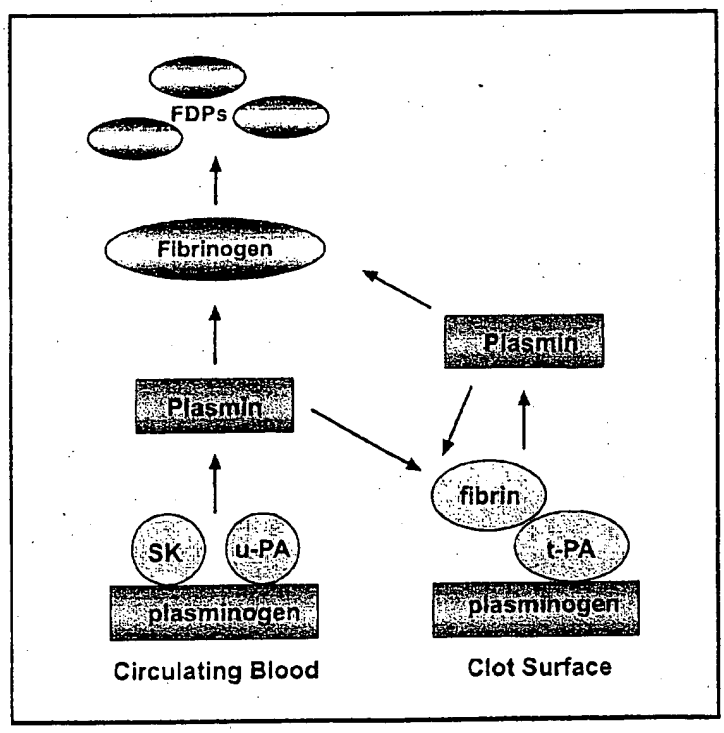

Fig. (3). Fibrinolysis. The fibrinolytic system is made up of the plasminogen, an inactive proenzyme, which may be converted to the active enzyme plasmin, which then degrades fibrin into fibrin degradation products (FDPs). Two physiologic plasminogen activators (PAs) exist; urokinase-type PA (u-PA) predominantly acting on circulating and cell-bound plasminogen, and tissue-type PA (t-PA), acting mainly on clot-bound plasminogen. Streptokinase (SK) acts mainly on circulating and cell-bound plasminogen.

Asn, and the sequence Lys 296-His-Arg-Arg is substituted for Ala-Ala-Ala-Ala, and Asn 117 is replaced by Gln [95]. These changes increase resistance to degradation and prolong half-life, but to date have not translated to clinical benefit over t-PA [96]. n-PA (lanoteplase) has a point mutation with Asn 117 being replaced by Gln 117, and the fingerlike and epidermal growth factor domains are deleted, which increases potency and prolongs half life, but again demonstrated only equivalence in terms of mortality benefit, with possibly increased risk of hemorrhage [95].

Other novel thrombolytics include bat t-PA and staphylokinase (SAK). SAK is 136 amino acid recombinant form of a PA naturally produced by *Staphylococcus aureus*. Like SK, antigenicity may be induced, but a number of variants have been produced to minimise this, and increase fibrin selectivity [97]. A version of one of these SAK derivatives is currently undergoing a dose and safety trial [98]. Bat PA was originally derived from the saliva of the vampire bat *Desmodus rotundus*, but has since been available as a recombinant protein [99]. It has greater fibrin specificity, but to date has not shown higher arterial patency, and may be associated with increased bleeding risk [100].

Given the only limited success with novel thrombolytics, efforts have more recently turned to combining thrombolytics with novel antiplatelet and antithrombotic agents [101]. This approach is attractive in that it provides potentially potent inhibition of fibrin, platelet aggregation and thrombus formation [102], although has the potential for increased bleeding risk. Early studies with full dose thrombolytic and the IIb/IIIa receptor antagonist eptifibatide were associated with increased bleeding [103], as did subsequent studies using half-dose thrombolytic and abciximab [104]. In contrast, half dose thrombolytic using TNK and the low molecular weight heparin derivative enoxaparin in an initial study appeared to provide benefit without increased risk [105].

NOVEL AND EMERGING THERAPIES FOR CHRONIC CARDIOVASCULAR DISORDERS

Heart Failure and Hypertension

The use of angiotensin converting enzyme (ACE) inhibitors has been central to the treatment of a wide spectrum of cardiovascular disease for over a decade. Initially used for the treatment of heart failure, these agents have since demonstrated benefit for patients with hypertension, left ventricular dysfunction and as agents reducing atherosclerosis progression [106]. This increased awareness of the importance of angiotensin as being involved in a number of pathogenic processes, including inflammation, plaque rupture and lesion progression, has lead to great interest in the possibility that angiotensin II receptor antagonists (or ARBs, angiotensin II receptor blockers) may provide additional benefits [106]. One such ARB, valsartan however showed no mortality reduction when added to ACE inhibitor therapy, although there were less hospitalizations and the suggestion of a minor degree of clinical benefit [107]. A second agent, telmisartan is currently being studied alone and in combination with the ACE inhibitor ramapril in 2 large clinical trials [108].

Endothelin Receptor antagonists are a second group of compounds targeting a separate physiologic pathway, with the potential for therapeutic use in a wide variety of cardiovascular disorders including hypertension and congestive heart failure. Endothelin (ET-1) is a naturally occurring peptide produced by intact endothelium. It is a physiologic antagonist of nitric oxide and a potent vasoconstrictor. It exists as 3 isoforms, ET1, 2 and 3, and likely acts on 2 receptors, A and B, although a third receptor may also exist [109, 110]. ET has had diverse roles proposed in the pathogenesis of hypertension, cardiac failure, myocardial infarction and coronary artery disease. ET antagonists have been tested in many of these settings principally using an antagonist of both A and B receptors, bosentan. For the most part these early clinical studies have demonstrated benefit in reduction of blood pressure and increased cardiac output and other indices of blood flow, though the physiologic significance of these effects remains incompletely understood, pending larger studies. Investigations are also underway for endothelin converting enzyme (ECE) inhibitors and similar drugs targeting neutral endopeptidase 24.11 (NEP), a related neurohumoral modulator [109, 110].

Despite the pathogenetic complexity and diffuse nature of hypertension, a number of investigators have also made efforts to treat this disorder using a gene therapy approach (reviewed in [111]). Various strategies have been attempted in the rat, using naked DNA or viral delivery systems targeting diverse genes including eNOS, atrial natriuretic peptide and kallikrein, resulting in modest transient lowering of systemic blood pressure. These are yet to proceed to human studies [111].

Chronic Ischemia and Angiogenesis

Chronic myocardial ischemia – lack of myocardial oxygenation due to insufficient blood supply – has traditionally been treated with mechanical attempts at revascularisation. Such methods have recently been improved, and also used in combination with novel pharmacologic therapies [112]. One such example is in the pharmacologic means of achieving angiogenesis.

Angiogenesis is the process by which new capillary vessels sprout and grow from existing vessels, as occurs in wound healing or at the edges of infarcted myocardial territory. In contrast, vasculogenesis refers to vascular growth from endothelial progenitor cells as occurs principally in embryonic development, leading to primitive blood vessel formation. Until recently, it was considered that vasculogenesis is confined to the embryonic phase of development. However, it has recently been shown that circulating endothelial precursor cells are capable of incorporating into regions of injury and ischemia and meaningfully contribute to increased vascularity [113, 114]. Arteriogenesis refers to the new appearance of fully-formed vessels, independent of their origin. It is generally considered that arteriogenesis reflects the remodeling of small vessels which are able to increase their radius and recruit smooth muscles to develop a media [115]; often, these remodeled new arteries take a cork-screw path through the tissue and are angiographically visible. The term neovascularization encompasses all three processes.

A multitude of growth factors and cytokines contributing to vascular growth have been identified [116]. It has been proposed that delivery of one or a group of these substances either by gene transfer or directly within ischemic myocardium will enhance the vascular growth process, improve myocardial perfusion and reduce symptoms of ischemia. Most pre-clinical and clinical studies have focused on the use of one of two cytokines: Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), and in many cases, naked plasmid DNA has been used rather than using viral vectors [117]. To date, the published clinical trials of angiogenesis for coronary or peripheral vascular disease have been largely limited to small, single center phase I or II trials [117, 118]. For coronary disease, 5 published trials have studied a total of 165 patients. These trials have focused on VEGF and FGF, in several isoforms, given as gene/vector combinations or protein, and using a number of delivery methods, for the most part in a non-randomized fashion. Overall, they have demonstrated feasibility and safety, and although not designed to do so, have also suggested efficacy. Novel techniques have also been used to achieve intramyocardial delivery via the percutaneous route. A preliminary report has demonstrated feasibility of delivering naked plasmid DNA for phVEGF-2 in humans [119].

NOVEL AND EMERGING THERAPIES IN PREVENTATIVE CARDIOLOGY

Dyslipidemias

Given the well-established links between dyslipidemia and subsequent cardiovascular events, dietary and pharmacologic interventions have been advocated in efforts to reduce risk [120]. A number of large randomized controlled trials have established the place of 3-hydroxy-3-methylyglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins in the treatment of dyslipidemias. Reductions of LDL-C in the range of 25-35% have consistently been shown with a variety of agents, translating to reductions of 24-37% in coronary events [121]. However, given the observations that most coronary events occur in patients with normal or only mildly elevated cholesterol, novel treatment strategies continue to be examined. Two such approaches are to use more potent agents to achieve further reductions in LDL-C, and to use novel agents targeting other lipid moieties [121].

Rosuvastatin is a novel statin with increased selectivity for activity within the liver, prolonged duration of action leading to increased ability to reduce LDL-C [122]. This agent is capable of dose-dependent reductions of LDL-C of 34 to 65% and is currently the subject of a randomized phase III trial compared to established statins [123]. NK-104 is a structurally similar agent also undergoing trial [124]. Other approaches in attempting greater LDL-C reductions include novel agents blocking other physiologic pathways [121]. Cholesterol transport inhibitors such as ezetimibe inhibit cholesterol absorption in the intestine and induce LDL-C reductions of nearly 20% [125]. Acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitors such as avasimibe may reduce macrophage cholesterol storage, although these agents are in general poorly absorbed, and have little effect on plasma cholesterol, making monitoring difficult [124].

Lastly, in populations with average total cholesterol and LDL-C levels, there is increasing awareness of the role of other lipid fractions; HDL-C appears as a predictor of future events, as well as apolipoproteins A-I (the major apolipoprotein in HDL) and apolipoprotein B (the major apolipoprotein in LDL) [121]. It is therefore possible that enzyme inhibition in these pathways may also have therapeutic use.

Dyslipidemias have also been subject to initial studies attempting gene transfer (reviewed in [111]). This has taken a number of strategies. One approach has been to increase high density lipoprotein (HDL), levels of which are inversely related to atherogenesis risk. The observation that ApoA-1 over-expressing transgenic mice have increased HDL levels, prompted efforts to over-express human ApoA-1 by adenoviral gene transfer, which results in a potent anti-atherogenic effect [126]. Other potential targets have been the LDL and VLDL receptors in the inherited dyslipidemias resulting from deficiencies in these. Autologous hepatocytes transfected with LDL receptor carrying virus have been transplanted in a pilot human study and resulted in reductions in circulating LDL levels, suggesting this approach may be feasible [111].

NOVEL AND EMERGING THERAPIES IN MALIGNANT HAEMATOLOGY

The Conventional Approach to the Treatment of Haematological Malignancies

The use of arsenicals in the treatment of leukaemia dates back at least to 1865 and the use of radiation therapy preceded World War I [127]. The haematopoeitic toxicity of mustard gas, observed with its use in World War I led to the development of a generation of alkylating agents, such as chlorambucil, busulphan and cyclophosphamide which are still in use today. The 1960s heralded the use of combination chemotherapy and adjuvant radiotherapy in the treatment of leukaemia and lymphoma with the realization that cures were possible in a subset of patients [128]. Since then, advances in antimicrobial therapy and transfusion medicine have complimented the higher cure rates achieved by the newer cytotoxic drugs and bone marrow transplantation in the treatment of leukaemia.

The diagnosis and classification of haematological malignancies, at present, is based predominantly on the morphology, immunophenotype and clinical behaviour of the malignant clone. Patients are stratified into risk categories based on patient and tumour characteristics foremost amongst which are the patient's age and the cytogenetic abnormality of the malignant clone, to guide therapy and prognosis. The majority of malignancies are treated at present with multiple cycles of chemotherapy and/or radiotherapy, followed when appropriate by autologous or allogeneic bone marrow transplantation.

The Promise of Microarray Technology in Tumour Diagnosis, Classification and Treatment

Carcinogenesis is a multistep process characterized by the acquisition of a series of genetic lesions, which confer a

survival advantage to the malignant clone. The particular combination of genetic lesions acquired by a tumour cell is probably unique and defines its clinical behaviour. It would also be expected that tumours with closely related genetic lesions would demonstrate a similar pattern of behaviour. A malignant clone can therefore be defined and categorized by its gene expression profile, and DNA microarray technology provides a mechanism by which this profile can be read, computed and interpreted.

The DNA microarray utilizes a series of mRNA or oligonucleotide probes, immobilized on a solid support, to survey sample cDNA. Sample cDNA is prepared by reverse transcription of mRNA extracts using fluorescent dyes. The labeled sample cDNA is mixed with standard cDNA (labeled with a different dye), and hybridized with the arrayed mRNA probes. The fluorescence emitted by sample and standard cDNA at each spot is quantified and expressed as a ratio. The density of mRNA probes on the solid support varies considerably, hence the use of standard cDNA to standardize results between arrays. The gene expression profile created for each sample can then be compared with others, to group samples with like profiles [129]. The clinical utility of gene expression profiling is still in its infancy. It has been demonstrated that tumours classified by conventional methods can also be differentiated by their gene expression profiles [130], [131]. More importantly, tumours, which appear to share a common cell of origin but respond differently to treatment, have been successfully subcategorized based on their gene expression profile. Despite these successes, the true potential for this technology lies not in replacing current methods of diagnosis and classification of tumours but in identifying new molecular targets for the design of novel agents. Furthermore, diagnostic microarrays spotted with genes that can be regulated by such designer drugs, could also be used to rationalize available therapy [132].

Designer Drugs

Cell proliferation, differentiation and apoptosis are highly regulated and surprisingly interrelated events. Each event is a coordinated response by a cell to an extracellular stimulus. A number of signal transduction pathways, used by cell surface receptors to transmit extracellular signals to the nucleus have been described. These pathways ultimately impact on genes, which control the passage of cells through cell division. Malignant cells often survive by acquiring defects in signal transduction pathways and the cell cycle program, which confer independence from or an abnormal response to external stimuli. The identification and interruption of a pathway that drives a malignant clone could therefore potentially terminate its growth.

Targeting Anomalies of the Signal Transduction Pathways (Fig. (4))

A number of mutations associated with the *ras* and Janus kinase (Jak) signal transduction pathways have been identified and drugs that target these signaling proteins are already in clinical trials. The *ras* family of signaling proteins can exist as inactive GDP or active GTP forms. The signal cascade is initiated by an extracellular ligand binding to a member of the receptor tyrosine kinase (RTK) family followed by autophosphorylation of the receptor. The signal

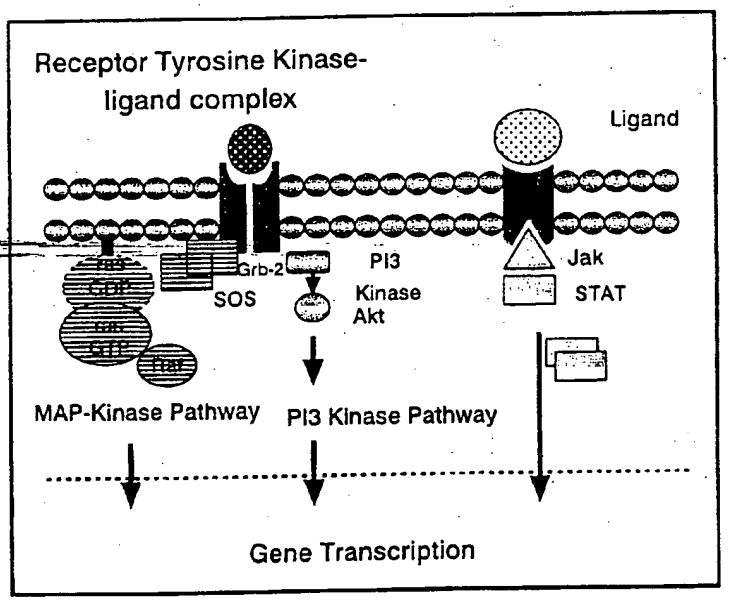

Fig. (4). Targeting the signal transduction pathway. The signal cascade is initiated by an extracellular ligand binding to a member of the receptor tyrosine kinase (RTK) family followed by autophosphorylation of the receptor. The signal is transduced from the cell surface to the gene regulatory machinery by sequential activation of proteins by phosphorylation, which leads eventually to the control of gene transcription. Farnesyl transferase is an enzyme that prenylates the *ras* proteins, permitting their attachment to the inner cytoplasmic membrane where they are positioned to participate in signal transduction. Members of the cytokine receptor superfamily lack intrinsic tyrosine kinase activity and rely on Jak mediated phosphorylation of the ligand–receptor complex for the subsequent binding and dimerization of Stat and the initiation of downstream signaling.

is transduced from the cell surface to the gene regulatory machinery by sequential activation of proteins by phosphorylation, which leads eventually to the control of gene transcription.

The therapeutic success of imatinib mesylate, formerly known as STI 571, in the treatment of chronic myeloid leukaemia (CML) bears testimony to the promise of targeted molecular based therapy. Imatinib was designed specifically to block the ATP binding site of the constitutively activated bcr-abl tyrosine kinase that is expressed in CML [133]. This drug has not only transformed the treatment of CML, but has heralded the advent of targeted molecular based therapy in mainstream haematology. Imatinib is not specific for the bcr-abl tyrosine kinase and has *in vitro* activity against mutant c-kit (stem cell factor receptor) cell lines and could have a broader application than originally intended. The crystal structure of a number of other normal and aberrant protein kinases has been determined permitting the rational design of drugs that occupy and block the active sites of these enzymes [134].

The *ras* family of proteins plays a pivotal role in signal transduction with far reaching downstream consequences in cell proliferation, differentiation and apoptosis. Farnesyl transferase is an enzyme that prenylates the *ras* proteins, permitting their attachment to the inner cytoplasmic membrane where they are positioned to participate in signal transduction. R115777 (tipifarnib), a farnesyl transferase inhibitor has completed phase I trials in acute myeloid leukaemia (AML) with an overall response rate of ~30% in relapsed and refractory AML [135].

Members of the cytokine receptor superfamily (interferon, erythropoeitin, granulocyte-colony stimulation factor etc) lack intrinsic tyrosine kinase activity and rely on Janus kinase mediated phosphorylation of the ligand–receptor complex for the subsequent binding and dimerization of signal transducer and activator of transcription (Stat) and the initiation of downstream signaling. Potential targets for drug design include, blocking Stat phosphorylation, interrupting the dimerization of Stat, interfering with the binding of Stat dimers to DNA binding domains and the use of oligonucleotide therapy to interfere with Stat expression [136], [137].

Targeting Mutant Transcription Factors

The signal that is transduced into the nucleus results in a change in the regulation of gene expression. This regulation is achieved by transcription factors that have DNA binding domains that recognize specific consensus nucleotide sequences and regulatory domains that directly or indirectly stimulate or repress gene expression.

A number of well recognized translocations in acute myeloid leukaemia result in the synthesis of aberrant transcription factors that often act as dominant negative inhibitors of the normal factor function.

The commonest molecular abnormality in acute promyelocytic leukaemia (APL) is the expression of a PML-RARα transcript as a result of a balanced translocation between chromosomes 15 and 17. Whereas the normal RARα (retinoic acid receptor α) transcript forms a heterodimer with members of the RXR (retinoic X receptor) family of transcription factors and activates gene transcription, PML-RARα recruits a nuclear co-repressor histone deacetylase complex and inhibits gene transcription. The introduction of all transretinoic acid (ATRA) in the treatment of APL has led to a dramatic improvement in survival. ATRA binds PML-RARα and reduces its affinity for the nuclear co-repressor complex and increases its affinity for transcription activators, effectively normalizing the transcription block imposed by the t (15; 17) translocation [138], [139], [140].

Two other translocations in AML, t (8; 21) and inv (16) are associated with the expression of fused transcription factors, AML1-ETO and CBFβ-MYH11 respectively, both of which inhibit gene transcription by recruiting nuclear co-repressor molecules and histone deacetylases [141], [142]. Histone deacetylase inhibitors (HDI) can reverse the block imposed by these mutant transcription factors but re-establishment of cell differentiation would require recruitment of co-activator molecules and histone acetylases to re-establish gene transcription. Nevertheless the efficacy of a number of HDI in inducing differentiation or apoptosis in malignant cell lines has led to the commencement of phase I trials in refractory multiple myeloma and non-Hodgkin Lymphoma using the HDI, Suberoylanalide hydroxamic acid (SAHA).

The paradigm for drug development in this area would be to first identify mutant transcription factors which are critical for the loss of cell differentiation and then modify them such that the co-activator complex is bound in preference to the co-repressor complex to re-establish gene transcription and cell differentiation.

Inhibitors of Cell Cycle Progression {Fig. (5)}

The passage of cells through the cell division cycle is a carefully orchestrated event. A cascade of protein phosphorylation pathways drive the cell through checkpoints that monitor completion of the molecular events and if necessary, delay progression to the next phase of the cycle. The cell cycle can be arbitrarily divided into G_1 (presynthetic), S (DNA synthesis), G_2 (premitotic) and M (mitotic) phases. The movement of cells from the quiescent phase, G_0, into the cell cycle is normally dependent on growth signals that are bypassed in malignancy. Major checkpoints exist between G_1-S and G_2-M.

The cyclin dependent kinases (cdk) are a family of constitutively expressed proteins that drive the cell cycle by the phosphorylation of groups of proteins that are required for cell division. The process is coordinated by the expression of growth phase specific cyclins that interact with and facilitate the activation of the cdks [143]. Cyclin D for example, is expressed when the cell enters the G1 phase, and interacts with cdks 4 and 6 to drive the cell across the G1-S checkpoint. The checkpoints are maintained by groups of cdk inhibitors and by suppressors such as the retinoblastoma protein, which sequesters growth promoting transcription factors. P53 is a commonly mutated tumour suppressor protein, which is expressed in response to stimuli known to damage DNA. P53 stalls the cell cycle at the G_2-M checkpoint by promoting the expression of p21, a cdk inhibitor that inhibits the CyclinB/CDK1 complex. The defect is then either repaired and the cell proceeds through

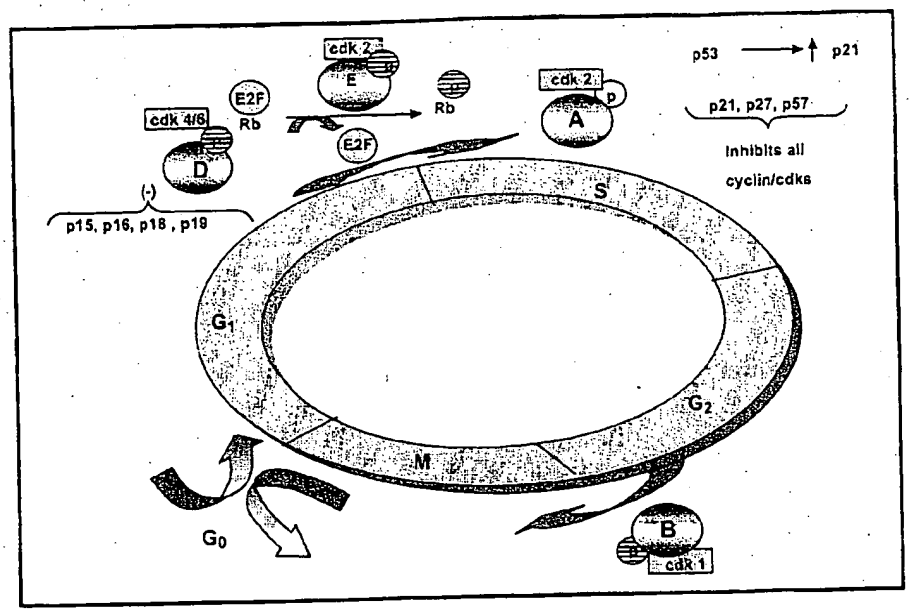

Fig. (5). Regulators of the cell cycle. The cell cycle can be arbitrarily divided into G_1 (presynthetic), S (DNA synthesis), G_2 (premitotic) and M (mitotic) phases. Major checkpoints exist between G_1-S and G_2-M. The process is coordinated by the expression of growth phase specific cyclins that interact with and facilitate the activation of constitutively expressed cyclin dependent kinases (cdks), which in turn phosphorylate proteins that govern cell division.

the cell cycle or the apoptotic program is activated and the cell dies [144]. As the cell progresses through the cycle the balance between the promoters and inhibitors is maintained by degradation of the cyclins and cdk inhibitors by the ubiquitin-proteasome pathway (vide infra). Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin lymphoma, characterized by the t (11; 14) translocation and the overexpression of Cyclin D1. Flavopiridol is a plant extract and the first inhibitor of cdks to enter clinical trials. It targets the ATP binding site of a number of cdks and is active against resting and proliferating cells. In phase 1 trials in MCL, flavipiridol appears to have limited efficacy as monotherapy. Inhibitors of Cyclin D1 that are designed to affect its binding to cdks, could in theory be more effective in MCL.

Inducers of Apoptosis

A mixture of growth, survival and death signals help maintain the density of the normal cell population. The pathway of programmed cell death or apoptosis is also triggered by pathological stimuli, and serves as a mechanism of deleting cells damaged beyond repair. The process involves the activation of the caspase family of proteolytic enzymes, which mediates the cleavage of cytoskeletal and nuclear proteins and the dissolution of DNA by the activation of cytoplasmic DNase. Caspase activity is regulated by adaptor proteins, which transmit signals from the cell surface and by the bcl-2 family of proteins. The bcl-2 family of proteins consists of pro and anti-apoptotic members, the balance of which determines the release of cytochrome c and other activators of the caspase family, across the outer mitochondrial membrane into the cytosol [145]. Fas (CD95) and TNFR1 (Tumour necrosis factor receptor 1) are cell surface receptors that are bound by soluble and surface bound ligands, and transmit death signals to the caspase family of effectors. TNF/TNFR1 interaction also promotes cell survival by the activation of the transcription factor NF-κB (Nuclear factor-κB) [146]. The apoptotic pathway can also be suppressed by survival signals, which alter the balance of anti-apoptotic bcl-2. The dead cells then demonstrate surface markers, which facilitate their recognition and removal by tissue macrophages.

The central role played by bcl-2 in regulating apoptosis and its over expression in a majority of follicular lymphomas and a proportion of diffuse large B cell lymphoma, chronic lymphocytic lymphoma, mantle cell lymphoma and multiple myeloma makes it an attractive target in drug development. Antisense oligonucleotides (ASO) directed against bcl-2 mRNA have completed phase 1 clinical trials. As with other potential targets for ASO therapy, an 18-mer-phosphorothiolated oligonucleotide, G3139, complementary to the first six codons of the open reading frame of the bcl-2 mRNA was designed. It is intended that the oligonucleotide, which has had its phosphodiester backbone modified to make it nuclease resistant, will form a DNA-mRNA heteroduplex with the bcl-2 transcript and activate RNaseH an enzyme that cleaves the mRNA moiety preserving the ASO. It is predicted that the combination of ASO and chemotherapy will, at least be additive, if not synergistic and a phase 1 trial involving G3139 and cyclophosphamide in relapsed follicular lymphoma has commenced [147], [148].

Perforin/granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to kill virus-infected or transformed cells [149]. It is proposed that a

macromolecular complex released by effector cells, composed of perforin and granzyme B, complexed to the lymphocyte granule proteoglycan, serglycin binds to the membrane of target cells and is endocytosed. Within the acidic vesicle, perforin disrupts the endocytic membrane releasing the granzyme B complex into the cytosol triggering apoptosis of the target cell [150]. Understanding the biophysical properties of this natural modular drug delivery system may provide insights into the utilization of its components in future drug design.

Gap junctional intercellular communication (GJIC) is thought to regulate cell growth, differentiation and apoptosis [151]. Impaired GJIC contributes to the growth and spread of cancer cells and restoring normal function would in theory help re-establish some growth control. The complex cell-cell and cell-matrix interactions required for normal GJIC, make this a difficult challenge. Blocking the tumour promoters that disrupt GJIC and contribute to malignant growth would be a more logical option than attempting to restore normal GJIC in neoplastic cells. Even this strategy would need to be part of a multifaceted approach to achieve meaningful tumour kill.

Inhibitors of the Proteasome

The proteasome is a multicentric protease complex that mediates the degradation of intracellular proteins and plays a critical role in the regulation of their function [152]. The proteins targeted for degradation are first conjugated with ubiquitin. Targets for the ubiquitin-proteasome pathway include proteins involved in cell cycle control, apoptosis, transcription factor activation and cell motility. Bortezomib, formerly known as PS 341 is a proteasome inhibitor that has entered clinical trials. By increasing the concentrations of these regulatory proteins, bortezomib appears to shift the balance away from cell survival to apoptosis. Multiple myeloma, CLL and mantle cell lymphoma are examples of malignancies that have acquired molecular defects that favour cell survival and impair apoptosis, which make the malignant clone particularly difficult to eradicate by conventional therapy [153]. The combination of cytotoxic therapy and bortezomib has promise and the outcome of these trials is eagerly awaited.

For the full potential of targeted molecular based therapy to be realized, the salient molecular defects that drive a particular malignant clone should first be identified. The development and application of gene expression profiling by microarrays will need to progress in parallel to contend with the increasing complexities of diagnosis and treatment. It is conceivable that with time, the most appropriate of the available molecular based therapies, will be picked with the aid of a computer algorithm based on the gene expression profile of a particular tumour.

Immunotherapy

Serotherapy

The initial concept of raising monoclonal antibodies against tumour specific antigens, for use in targeting cytotoxins to the tumour and avoiding collateral damage to normal cells has been modified by a number of practical issues. Developing unique antibodies is an expensive and time-consuming process and in the case of a rapidly proliferating B cell lymphoma, possibly futile and even dangerous. Tumour specific idiotypic antibodies on the surface of B cell lymphomas would seem at first an ideal target for the development of monoclonal antibodies, but as the antigen (in this instance an idiotypic antibody) is shed into the circulation in large quantities, the monoclonal is neutralized before it reaches its target with the added problem of immune complex formation and tissue deposition.

The trend therefore has been to develop both conjugated and unconjugated humanized monoclonal antibodies against common tumour antigens. The ideal antigen would be densely expressed on the surface of the tumour relative to normal cells, would not be shed into the bloodstream and would not be internalized following antibody binding. The ideal antibody would reach and bind the target antigen with high avidity, would not induce host anti-monoclonal alloantibodies, and would effectively activate the complement cascade, induce apoptosis and bind macrophages, killer and NK cells to eliminate the target tumour cell.

There is now considerable clinical experience with the use of Rituximab, an anti-CD 20 humanized mouse monoclonal antibody in a wide variety of B-Cell lymphomas [154]. It has proved most effective in the treatment of indolent non-Hodgkin's lymphomas, which express high titres of CD 20, although its role in aggressive lymphomas both as single therapy and in conjunction with chemotherapy is being explored. As expected there is a depletion of normal B-lymphocytes from the circulation but as plasma cells do not express CD 20, serum immunoglobulin levels are maintained during Rituximab therapy.

Campath-1H, a humanized anti CD 52 IgG 1, is licensed for use in patients with refractory chronic lymphocytic leukaemia. As CD 52 is expressed in a wide variety of immunocompetent cells, including B and T lymphocytes, monocytes and NK cells, it is not surprising that a profound immunodeficiency results and prophylactic therapy for pneumocystis and herpes simplex virus is often commenced with Campath-1H therapy.

HuM195 is a humanized anti CD 33 monoclonal antibody designed for use in acute myeloid leukaemia (AML) in which more than 90% of blasts express CD33, a pan myeloid marker [155]. Although HuM195 results in myelosuppression, this effect is transient as CD 33 negative stem cells reconstitute the myeloid cell lines. As an unconjugated antibody, anti-CD 33 has limited clinical efficacy, but conjugated with an antibiotic, calicheamicin, and licensed as Mylotarg, it has proved effective and has been approved for treating relapsed CD33 positive AML in elderly patients who are not candidates for aggressive remission induction therapies.

Radioimmunoconjugates, combining γ or β particle emitters with monoclonal antibodies, have entered clinical trials in the treatment of refractory and relapsed lymphoma [156]. The radioimmunoconjugates have the theoretical advantage of targeting therapy to antigen positive tumour cells and killing antigen negative mutants and surrounding

cells that are inaccessible to the antibodies, by a radioactive field. I^{131}-immunoconjugates are cheap and effective and can be used for both imaging and treatment, but pose a radiation hazard to associates of the treated individual. β Particle emitters such as Y^{90} do not have this drawback but require surrogate radioisotopes to establish biodistribution. Anti-CD33 radioisotope conjugates have proven too toxic for therapy but may have a role in conditioning regimens for bone marrow transplantation by allowing the administration of a higher dose of radiation to the bone marrow, relative to normal tissue.

Cellular Therapy

The very survival of tumour cells reflects, in a sense, a failure of the host's immune system to recognize and eliminate abnormal tissue. The failure of the immune system to eliminate tumour cells is variably attributed to the decreased immunogenicity of these cells and the immunosuppressive environment generated by the tumour. Efforts therefore have focused on mechanisms by which this induced tolerance might be overcome. Tumour vaccines based on antigen-loaded dendritic cells have entered clinical trials [157, 158]. Dendritic cells have limited phagocytic capacity but are highly effective antigen presenting cells [159]. Immature dendritic cells can be harvested from peripheral blood by leukopheresis and separated by density centrifugation or grown from haemopoeitic stem cells or monocytes. There are unfortunately very few clinically useful tumour specific antigens and the prospect of generating vaccines for individual tumours is impractical. The focus therefore has shifted from tumour specific antigens to the use of tumour associated and tissue specific antigens. Telomerase, for example, is expressed in a majority of tumours and rarely if at all by normal adult cells and is therefore a potential antigen for tumour vaccines [160]. The use of tissue specific antigens such as prostatic acid phosphatase in tumour vaccines could potentially result in damage to both normal and tumour cells, but the damage would at least be limited to a particular tissue type. The selected antigen is usually engineered to optimize uptake and processing by dendritic cells. Mature dendritic cells that express the antigen and demonstrate co-stimulatory molecules are reinfused to the host with the intent that they induce a T cell driven immune response against the resident tumour. Idiotype pulsed dendritic cells have been used in the treatment of B-cell lymphoma and multiple myeloma with encouraging results.

A related approach has been the use of activated polyclonal or antigen specific autologous T cell infusion [161]. In the antigen specific T cell approach, antigen loaded dendritic cells or MHC-peptide tetramer complexes are used to select, activate and expand autologous T cells, which are then reinfused to the patient.

Encouraged by the success of donor T-lymphocyte infusion (DLI) in the treatment of relapsed CML, the graft versus leukaemia (GVL) effect of allogeneic T cells has been harnessed in the use of allogeneic stem cell transplantation after non-myeloablative conditioning to treat a variety of haematological and solid malignancies. Graft versus host disease (GVHD) is a dose related complication of DLI. There is early evidence to suggest that $CD4^-$ DLI may retain its GVL effect with a lower incidence of GVHD [162].

Anti-angiogenic Drugs

The haemangioblast is the common progenitor of both vascular endothelial cells and haematopoeitic stem cells. A small proportion of $CD34^-$ stem cells, express VEGFR-2 (vascular endothelial growth factor receptor-2), and has the potential to reconstitute both haematopoeitic cells and the vascular endothelium [163, 164]. The shared lineage between haematopoeitic cells and endothelial cells permit certain malignant haemopoeitic cells to secrete and respond to angiogenic factors.

Angiogenesis is a coordinated process, mediated in the main by a family of vascular endothelial growth factors (VEGF) that regulate the breakdown of the vascular basement membrane, dissolution of the surrounding matrix, proliferation and migration of endothelial cells and the formation of a capillary lumen. The process is completed by reconstitution of the basement membrane and termination of endothelial cell proliferation [165].

We have developed a peptide trivalent arsenical, 4-(*N*-(*S*-glutathionylacetyl)-amino) phenylarsenoxide (GSAO), that is a remarkably effective inhibitor of tumour angiogenesis and tumour growth in mice [166]. Moreover, it is orally available and has no side-effects at efficacious doses. GSAO triggers apoptosis of proliferating but not quiescent endothelial cells in culture and is selective for endothelial cells compared to other cell types. The ease of synthesis, small size, apparent safety and oral availability of GSAO bodes well for its potential as a therapeutic for the treatment of human cancer and other angiogenesis-dependent diseases.

More than 40 endogenous anti-angiogenic compounds have been described. Attempts to use these compounds in clinical trials have been hindered by high dose requirements and the relative instability of the recombinant proteins. The focus has shifted to the manufacture of synthetic anti-angiogenic compounds and anti-angiogenic gene therapy [167, 168]. AG3340 (Prinomastat) is a gelatinase inhibitor in phase II trials in the treatment of patients with the myelodysplastic syndrome (MDS). Its anti-angiogenic properties relate to the inhibition of basement membrane dissolution and endothelial cell migration.

A number of inhibitors of endothelial cell activation and proliferation are currently in clinical trials. Bevacizumab is a humanized anti VEGF monoclonal antibody, in phase II trials in MDS, AML and lymphoma [169]. Thalidomide and its analogues, in addition to their direct cytotoxic effect, inhibit βFGF and VEGF induced angiogenesis and is effective in the treatment of multiple myeloma and is being trialled in the treatment of MDS [170]. The VEGF receptors belong to the receptor tyrosine kinase family and utilize the *ras* signaling pathway for signal transduction. A number of receptor tyrosine kinase inhibitors, which have broad receptor specificity, are in clinical trials [171].

A number of endogenous anti-angiogenic compounds have been used in gene therapy models in experimental animals with a degree of success. Short of intratumoral injection of the vector, targeted gene therapy remains elusive. As a consequence, normal host tissue is used to express these compounds at supra physiological concentrations. It is hoped that the low toxicity profile

observed in animal experiments will translate to humans without loss of efficacy [168].

Gene Therapy

Gene therapy was initially conceived as a means of treating single gene disorders but contrary to this original dictum; cancer, which is usually characterized by a multiplicity of genetic mutations, has become the commonest application for gene therapy in clinical trials. These clinical trials have by and large addressed the treatment of solid tumours but have parallels in the treatment of haematological malignancies. The basic approaches have revolved around the induction of tumour immunogenesis, suicide gene therapy and redressing defects in the cell cycle program [172].

The most frequently used approach in tumour immunogenesis, is the intratumoral injection of fibroblasts engineered to express cytokine genes such as IL-2, Interferon γ or TNF α. Suicide gene therapy involves the introduction of genes, which express enzymes such as herpes simplex virus thymidine kinase that convert non-toxic drugs to cytotoxic compounds. Mutations in p53 are noted in ~ 50% of certain solid tumours. Gene transfer of wild type p53 to a wide variety of tumors has yielded only limited success [173]. The published results of the many clinical trials in gene therapy in cancer have in general been disappointing. The need for intratumoral injection of the vector remains a major drawback. Methods of targeting gene therapy to the tumour, short of intratumoral injection, will need to advance in parallel with the advances made in vector design, to derive benefit from gene therapy in cancer.

Haemopoeitic Cytokines

The demonstration in the early 1960s, that cells in the bone marrow have the capacity to establish haemopoeitic colonies of multiple lineages in the spleen when injected into irradiated mice [174], helped advance the concept of stem cells resident in the bone marrow regenerating the various blood cell lines in a regulated manner. The purification of colony stimulating factors that favour the proliferation of one cell line over another followed a couple of decades later [175]. It is now clear that none of the colony stimulating factors are completely lineage specific and that the most primitive cells require the highest number of regulators for cell multiplication and differentiation [176]. Erythropoeitin, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), thrombopoeitin and stem cell factor are used in clinical practice to a variable degree but the appropriate indications for their use, the most useful combination and the best timing are still debated despite many years of clinical experience.

As an alternative to blood transfusion, erythropoeitin has an established role in the treatment of anaemia associated with chronic renal failure. There is also compelling evidence for its use in anemic patients with lymphoproliferative disorders who are receiving chemotherapy [177]. Although used in anemia associated with myelodysplasia, the best response appears to be in combination with G-CSF [178-180] and in patients with a low serum erythropoeitin level and a baseline red-cell transfusion requirement of less than a couple of units per month [181]. Evidence based guidelines are being formulated by the American Society of Hematology and the American Society of Clinical Oncology which should help clarify some of these outstanding issues. A recent report of pure red-cell aplasia and anti-erythropoietin antibodies in 13 patients treated with recombinant erythropoeitin is alarming but should be considered against the estimated 3 million patients worldwide who currently receive erythropoietin [182]. 6 of these 13 patients recovered some endogenous erythropoeitin function with cessation of the recombinant erythropoietin therapy. The long acting formulation, darbepoetin alfa, can be administered weekly instead of the conventional twice or thrice weekly regimen and should prove useful in the clinic.

Randomized controlled studies support the use of G-CSF to limit the interval of neutropaenia and neutropaenia associated febrile episodes following chemotherapy and bone marrow transplantation, but without a survival advantage from its use. There is also no clear evidence that G-CSF stimulates early re-growth of the malignant clone at the doses and schedules that are currently used in clinical practice, but concerns linger particularly with its use in neutropaenia associated with MDS [183]. The use of G-CSF in mobilizing haemopoeitic stem cells for peripheral collection has greatly benefited bone marrow transplantation.

Two forms of thrombopoietin have entered clinical trials. rhTPO is a recombinant protein of the native molecule and PEG-rHuMGDF (megakaryocyte growth and differentiation factor) is a PEGylated recombinant protein of a truncated version. Several clinical trials have demonstrated early platelet recovery associated with the administration of thrombopoietin a few days prior to nonablative myelosuppressive treatments but appears to be less effective in stem cell transplantation where the need for early platelet recovery is greater (reviewed in [184]). The early report of the development of neutralizing anti-TPO antibodies following the administration of PEG-rHuMGDF to normal platelet pheresis volunteers has been followed by a report of pancytopaenia associated with anti-TPO antibodies after multicycle chemotherapy supported by MGDF in a patient with ovarian carcinoma [185].

Stem cell factor is currently marketed for use in conjunction with G-CSF to mobilize peripheral haemopoeitic stem cells but other clinical applications for its use are being explored. The allergic reactions associated with mast cell degranulation and melanocyte proliferation at the site of injection have tempered its use but SCF analogs without these deleterious effects are under development. Both TPO and SCF are used in the various cytokine combinations that are under investigation in the ex-vivo expansion of transplantable stem cells (reviewed in [186]).

There is increasing evidence of the complexity of growth factor actions. GM CSF may promote myocardial coronary collateral formation, and play roles in macrophage metabolism within atherosclerotic lesions [187]. As this, and related agents undergo further evaluation, their wide-ranging activities may have implications far beyond their initial application, and may again provide examples of convergence between the fields of haematology and cardiology.

NOVEL AND EMERGING THERAPIES FOR THE COMMON CHRONIC HAEMATOPATHOLOGIES

Haemophilia

The sex-linked inheritance of Haemophilia A and Haemophilia B, was recognized in the 19th century but its distinction not made till 1952. With advances in plasma fractionation and chromatography, increasingly pure preparations of Factor VIII and IX have gradually replaced fresh frozen plasma and cryoprecipitate in the treatment and prophylaxis of bleeding associated with haemophilia. In countries that can afford the high cost, recombinant Factor VIII and IX therapy is gradually replacing plasma derived preparations as the standard, in the treatment of newly diagnosed cases of haemophilia. Recombinant Factor VIIa is licensed for use in patients with haemophilia who develop inhibitors to factors VIII or IX and has also proved useful in stemming haemorrhage in massively transfused non-haemophilia patients.

Haemophilia B is an attractive model for gene therapy. The clinical phenotype can be altered by circulating levels of factor IX >1%, which accommodates the low expression observed with most gene therapy models. Furthermore, the gene and protein are well characterized and there are animal models for the disease. The commencement of a clinical trial using a liver directed adeno associated virus (AAV)/human factor IX construct in patients with haemophilia B is a landmark in gene therapy [188]. It is hoped that the development of factor IX inhibitors will be no greater than in recipients of plasma derived or recombinant factor IX.

Thalassaemia and Sickle Cell Anaemia

The tertiary and quaternary structure of haemoglobin and its relevance in a number of heritable disorders such as sickle cell anaemia and thalassaemia have been known since the 1940's and 50's but with the exception of prenatal testing and the use of iron chelation therapy few advances have been made which benefit the majority [189]. The secondary iron overload associated with chronic transfusion therapy in severe thalassaemia and sickle cell anemia can be reversed by the prolonged administration of subcutaneous or intravenous deferoxamine (DFO) although long term compliance is difficult to maintain. Protocols for alternate regimens to improve patient compliance are under investigation and a report that twice daily SC injection achieves the same urinary clearance as does prolonged SC infusion is encouraging [190]. Although Deferiprone, an oral iron chelator is superior to erratic compliance with parenteral DFO or no therapy at all, it is an inefficient chelator and its association with agranulocytosis mandates regular blood count monitoring with its use [191]. A phase II study of another oral iron chelator ICL670 is now complete and this promising drug will soon enter a multicenter randomized phase III trial to compare its performance against parenteral DFO.

Primary surveillance for the prevention of strokes in sickle cell anemia using trans-cranial Doppler (TCD) to commence regular blood transfusion in those at risk, is of proven value. Whether hydroxyurea, which increases circulating HbF levels and reduces painful crises in sickle cell anaemia, is a viable alternative to regular blood transfusion as a stroke prophylactic in a subset of patients, remains to be tested.

NOVEL AND EMERGING THERAPIES USING STEM CELLS IN CARDIOLOGY AND HAEMATOLOGY

The rejection of transplanted tissue remains a major hurdle to the wider application of allogeneic haemopoeitic stem cell transplantation. Therapeutic cloning or nuclear transfer technology involves the transfer of differentiated recipient nuclei into a donor blastocyst with the express purpose of developing and harvesting embryonic stem cells that can be cultured ex-vivo to generate tissues expressing recipient histocompatibility antigens. The coupling of gene transfer technology to therapeutic cloning can, in theory, permit the transplantation of engineered stem cells to correct

Table 1. Innovative Technologies and their Overlapping Applications in Cardiology and Haematology

Technology	Current applications in cardiology	Current applications in haematology
Gene transfer	Restenosis Hyperlipidemias Heart Failure Hypertension	Haemophilia B Severe Combined Immunodeficiency (SCID)
Gene modification	Restenosis (Ribozyme, DNAzyme, Decoys) Vein graft patency (Decoys)	Lymphoma (anti-bcl-2 mRNA oligonucleotides (G3139)
Anti-proliferative drugs with molecular targets	Restenosis (Sirolimus, paclitaxel) Post transplant vasculopathy (Sirolimus)	Chronic myeloid leukaemia (Imatinib[1]) Multiple myeloma (SAHA[2], Bortezomib[3]), Non-Hodgkin lymphoma (SAHA[2], Bortezomib[3]) Chronic lymphocytic leukaemia (Bortezomib[3]) Acute myeloid leukaemia (Tipifarnib[4])
Stem cell technology	Heart Failure (cellular cardiomyoplasty)	Haematopoeisis (autologous/allogeneic haemopoeitic stem cells) SCID (gene transfer to autologous haemopoeitic stem cells)

[1]tyrosine kinase inhibitor; [2]histone deacetylase inhibitor; [3]proteasome inhibitor; [4]farnesyl transferase inhibitor

a number of single gene haematological disorders [192]. Ex-vivo gene transfer to autologous haemopoeitic stem cells could achieve the same end but would require a high rate of transfection and protein expression to be clinically meaningful. A recent report of ex-vivo gene transfer of the deficient γ_c chain to cure X-linked severe combined immunodeficiency was very encouraging [193]. However this and other related gene transfer programmes have been temporarily halted owing to the unfortunate development of T cell leukaemia in one of ten patients in the French trial [194]. This has been ascribed to the retroviral vector, the mouse Moloney leukemia virus, used in this study causing an insertional mutagenesis event in the host genome. Given the intense public debate surrounding the use of therapeutic cloning in research, it is clear that there are many scientific and ethical hurdles that need to be overcome before therapeutic cloning enters clinical practice. The use of embryonic stem cells to regenerate damaged tissue holds promise but is an application still in its infancy.

Analogous studies which again demonstrate the convergence between haematology and cardiology treatments, have used adult rat bone marrow haemopoietic stem cells and mesenchymal cells and demonstrated repopulation of infarcted rat myocardium. These cells differentiate into both cardiomyocyte and new blood vessels within myocardial tissue, suggesting that so-called cellular cardiomyoplasty (CCM) might be feasible, and is currently an area of intense research interest [195].

CONCLUSION

As the extent of the biological complexity of cell growth and regulation is understood, the unbridled enthusiasm at the dawn of the molecular era has now been tempered by a sense of reality. From current evidence, it is likely that many drugs under development that target a particular molecular defect may prove ineffective alone and will probably need to be used in combination with cytotoxics in current use to achieve disease remission. The production and marketing of drugs is a time consuming and expensive exercise. Commerce and market forces, not altruism, drive the pharmaceutical industry. It is therefore a reality that unless the number of potential consumers that can afford the drug reaches a critical mass, many worthy candidate drugs will not and cannot be championed by the pharmaceutical industry alone. This reality led the U.S. Food and Drug Administration to create the Office of Orphan Products Development in 1982 to administer the provisions of the Orphan Drug Act and the Orphan Products Grants Program which provide incentives for sponsors to develop products for rare diseases. Similar programmes are in place in a number of other developed countries as well.

Deciphering the human genome was not only a triumph for molecular biology but also a corridor to the immense power of computer science in the management and retrieval of biological data. The identification, analysis, comparison, prediction of structure and posttranslational modification of proteins has been greatly simplified by the availability of computer algorithms. But this is just the beginning. This wealth of information needs to be deciphered and form the basis for drug target identification, rational drug design, prediction of drug toxicity, pharmacokinetics and pharmacodynamics.

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REFERENCES

[1] Lane, D.M. Dramatic increase in the use of coronary stents. *American Journal of Cardiology*, 1999, *84*, 1141.

[2] Schwartz, R.S. In *Textbook of Interventional Cardiology*, Topol E.J., Ed.; WB Saunders: Philadelphia, 1994; pp.365-81.

[3] Lowe, H.C., Oesterle S.N., Khachigian L.M. Coronary in-stent restenosis: current status and future strategies. *Journal of the American College of Cardiology*, 2002, *39*, 183-93.

[4] Lowe, H.C., Fahmy, R.G., Kavurma, M.M., Baker, A., Chesterman, C.N., Khachigian, L.M. Catalytic oligodeoxynucleotides define a critical regulatory role for Early Growth Response Factor-1 in porcine coronary in-stent restenosis. *Circ. Res.*, 2001, *89(8)*, 670-7.

[5] DeYoung, M.B., Dichek, D.A. Gene therapy for restenosis. Are we ready? *Circulation Research*, 1998, *82*, 306-13.

[6] Libby, P. Gene therapy for restenosis. Promise and perils. *Circulation Research*, 1998, *82*, 404-6.

[7] Rome, J.J., Shayani, V., Flugelman, M.Y., Newman, K.D., Farb, A., Virmani, R., Dichek, D.A. Antaomic barriers influence the distribution of *in vivo* gene transfer into the arterial wall: Modeling with microscopic tracer particles and verification with a recombinant adenoviral vector. *Arterioscler Thromb.*, 1994, *14(1)*, 148-61.

[8] Verma, I.M. *Gene Therapy Scientific American*, 1990, *263*, 68-72.

[9] Losordo, D.W., Vale, P.R., Symes, J.F., Dunnington, C.H., Esakoff, D.D., Maysky, M., Ashare, A.B., Lathi, K., Isner, J.M. Gene therapy for myocardial angiogenesis: initial results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*, 1999, *98*, 2800-2804.

[10] Losordo, D.W., Vale, P.R., Hendel, R.C., Milliken, C.E., Fortuin, F.D., Cummings, N., Schatz, R.A., Asahara, T., Isner, J.M. Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation*, 2002, *105*, 2012-8.

[11] Manninen, H.I., Makinen, K. Gene therapy techniques for peripheral arterial disease. *Cardiovascular and Interventional Radiology*, 2002, *25*, 98-108.

[12] Klugherz, B.D., Jones, P.L., Cui, X., Chen, W., Meneveau, N.F., DeFelice, S., Connolly, J., Wilensky, R.L., Levy, R.J. Gene delivery from a DNA controlled-release stent in porcine coronary arteries. *Nat. Biotechnol.*, 2000, *18(11)*, 1181-4.

[13] Ledly, F.D. Pharmaceutical approach to somatic gene therapy. *Pharm. Res.*, 1996, *13*, 1595-1614.

[14] Nabel, E.G., Nabel, G.J. Complex models for the study of gene function of in cardiovascular biology. *Annu. Rev. Physiol.*, 1994, *56*, 741-61.

[15] Sinnaeve, P., Varenne, O., Collen D. Gene therapy in the cardiovascular system: an update. *Cardiovascular Research*, 1999, *44*, 498-506.

[16] Varmus, H. Retroviruses. *Science*, 1988, *240*, 1427-35.
[17] Nabel, E.G., Plautz, G., Boyce, F.M., Stanley, J.C., Nabel, G.J. Recombinant gene expression *in vivo* within endothelial cells of the arterial wall. *Science*, 1989, *244*, *1342-1344*.
[18] Brieger, D., Topol, E.J. Local drug delivery systems and prevention of restenosis. *Cardiovascular Research*, 1997, *3*, 405-13.
[19] Wilensky, R.L., March, K.L., Gradus-Pizlo, I., Schauwecker, D., Michaels, M.B., Robinson, J., Carlson, K., Hathaway, D.R. Regional and arterial localisation of radioactive microparticles after local delivery by unsupported and supported porous balloon catheters. *Am. Heart J.*, 1995, *129(5)*, 852-9.
[20] Teiger, E., Deprez, I., Fataccioli, V.,Champagne, S., Dubois-Rande, J.L., Eloit, M., Adnot, S. Gene therapy in heart disease. *Biomedical. Pharmacotherapies*, 2001, *55*, 148-54.
[21] Laitinen, M., Hartikainen, J., Hiltunen, M.O. Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. *Human gene therapy*, 2000, *11*, 263-70.
[22] O'Sullivan, M., Bennett, M.R. Gene therapy for coronary restenosis: is the enthusiasm justified? *Heart*, 2001, *86*, 491-3.
[23] Khachigian, L.M. Catalytic DNAs as potential therapeutic agents and sequence-specifica molecular tools to dissect biologic function. *J. Clin. Invest.*, 2000, *106(10)*, 1189-95.
[24] Hogrefe, R.I. An antisense oligonulceotide primer. *Antisense Nucleic Acid Drug Dev.*, 1999, *9(4)*, 351-7.
[25] Simons, M., Edelman, E.R., DeKeyser, J.L., Langer, R. Antisense c-myb oligonucleotides inhibit intimal smooth muscle cell accumulation *in vivo*. *Nature*, 1992, *359*, 67-73.
[26] Gunn, J., Holt, C.M., Francis, S.E., Shepherd, L., Grohmann, M., Newman, C.M., Crossman, D.C., Cumberland, D.C. The effect of oligonucleotides to c-myb on vascular smooth muscle cell proliferation and neointima formation after porcine coronary angioplasty. *Circulation Research*, 1997, *80*,520-31.
[27] Kipshidze, N.N., Kim, H.S., Iversen, P., Yazdi, H.A., Bhargava, B., New, G., Mehran, R., Tio, F., Haudenschild, C., Dangas, G., Stone, G.W., Iyer, S., Roubin, G.S., Leon, M.B., Moses, J.W. Intramural coronary delivery of advanced antisense oligonucleotides reduces neointimal formation in the porcine stent restenosis model. *J. Am. Coll. Cardiol.*, 2002, *39*, 1686-91.
[28] Kutryk, M.J., Foley, D.P., van den Brand, M., Hamburger J.N., van der Giesen, W.J., deFeyter, P.J., Bruining, N., Sabate, M., Serruys, P.W. Local intracoronary administration of antisense oligonucleotide against c-myc for the prevention of in-stent restenosis: results of the randomized investigation by the Thoraxcenter of antisense DNA using local delivery and IVUS after coronary stenting (ITALICS) trial. *J. Am. Coll. Cardiol.*, 2002, *39*, 281-7.
[29] Muotri, A.R., da Veiga Pereira, L., dos Reis Vasques, L., Menck, C.F. Ribozymes and the anti-gene therapy: how a catalytic RNA can be used to gene function. *Gene*, 1999, *237(2)*, 303-10.
[30] Macejak, D.G., Lin, H., Webb, S. Adenovirus-mediated expression of a ribozyme to c-myb mRNA inhibits smooth muscle cell proliferation and neointima formation *in vivo*. *Journal of Virology*, 1999, *73*, 7745-51.
[31] Yamamoto, K., Morishita, R., Tomita, N., Shimozato, T., Nakagami, H., Kikuchi, A., Aoki, M., Higaki, J., Kaneda, Y., Ogihara, T. Ribozyme oligonucleotides against transforming growth factor-B inhibited neointimal formation after vascular injury in the rat model. *Circulation*, 2000, *102*, 1308-14.
[32] Frimerman, A., Welch, P.J., Jin, X., Eigler, N., Yei, S., Forrester, J., Honda, H., Makkar, R., Barber, J., Litvack, F. Chimeric DNA-RNA hammerhead ribozyme to proliferating cell nuclear antigen reduces stent-induced stenosis in a porcine coronary model. *Circulation*, 1999, *99(5)*, 697-703.
[33] Li Y., Breaker, R.R. Deoxyribozymes: new players in the ancient game of biocatalysis. *Current Opinion in Structural Biology*, 1999, *9*, 315-23.
[34] Santiago, F.S., Lowe, H.C., Kavurma, M.M, Chesterman, C.N., Baker,A., Atkins,D.G., Kachigian,L.M. Novel DNA enzyme targeting NGFI-A mRNA inhibits vascular smooth muscle proliferation and regrowth after injury. *Nature Medicine*, 1999, *11*, 1264.
[35] Lunnon, M.W., Braddock, M. The impact of molecular medicine upon early cardiovascular drug development. *British Journal of Clinical Pharmacology*, 2001, *50*, 1-8.
[36] Morishita, R., Aoki, M., Kaneda, Y., Ogihara, T. Gene therapy in vascular medicine: recent advances and future perspectives. *Pharmacology and Therapeutics*, 2001, *91*, 105-14.
[37] Mann, M.J., Whittlemore, A.D., Donaldson, M.C., Belkin, M., Conte, M.S., Polak, J.F., Orav, E.J., Ehsan, A., Dell'acqua, G., Dzau, V.J. Ex vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single centre, randomised, controlled trial. *Lancet*, 1999, *354*, 1493-8.
[38] Keelan, P.C., Miyauchi, K., Caplice, N.M., Ashai, K.H., Schwartz, R.S. Modification of molecular events in coronary restenosis using coated stents: The Mayo Clinic Approach. *Seminars in Interventional Cardiology*, 1998, *3-4*, 211-5.
[39] Regar, E., Sianos, G., Serruys, P.W. Stent development and local drug delivery. *British Medical Bulletin*, 2001, *59*, 227-48.
[40] Drachman, D., Rogers, C. In *Coated Stents*, Rothman M. Ed.; Remedica: London, 2001: pp. 117-128.
[41] Serruys, P.W., Regar, E., Carter, A.J. Rapamycin eluting stent: the onset of a new era in interventional cardiology. *Heart*, 2002, *87*, 305-7
[42] Bennett, J.S. Novel Platelet Inhibitors. *Annu. Rev. Med.*, 2001, *52*, 161-84.
[43] EPIC investigators. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. *New England Journal of Medicine*, 1994, *330*, 956-61.
[44] Epilog investigators. Platelet glycoprotein IIb/IIIa receptor blockade and low-dose heparin during percutaneous coronary revascularization. The EPILOG Investigators. *New England Journal of Medicine*, 1997, *336*, 1689-96.
[45] EPISTENT investigators. Randomised placebo-controlled trial to assess safety of coronary stenting with use of platelet glycoprotein-IIb/IIIa blockade. The Epistent investigators. Evaluation of Platelet IIb/IIIa Inhibitor for stenting. *Lancet*, 1998, *352*,87-92.
[46] CAPTURE investigators. Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina. *Lancet*, 1997, *349*, 1429-35.
[47] Neumann, F.J., Blasini, R., Schmitt, C., Alt, E., Dirschinger, J., Gawaz, M., Kastrati, A., Schomig, A. Effect of glycoprotein IIb/IIIa receptor blockade on recovery of coronary flow and left ventricular function after the

placement of coronary-artery stents in acute myocardial infarction *Circulation*. 1998, *98*, 2695-701.

[48] Tam, S.H., Sassoli, P.M., Jordan, R.E., Nakada, M.T. Abciximab (Reopro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and alpha (v)beta3 integrins. *Circulation*, 1998, *98*, 1085-91.

[49] Harrington, R.A. Design and methodology of the PURSUIT trial: evaluating eptifibatide for acute ischemic coronary syndromes. Platelet glycoprotein IIb-IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy. *American Journal of Cardiology*, 1997, *80*, 34B-38B.

[50] PRISM investigators. A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. *New England Journal of Medicine*, 1998, *338*, 1498-505.

[51] O'Neill, W.W., Serruys, P.W., Knudtson, M., van Es, G.A., Timmis, G.C., van der Zwaan, C., Kleiman, J., Gong, J., Roecker, E.B., Dreiling, R., Alexander, J., Anders, R. Long-term treatment with a platelet glycoprotein-receptor antagonist after percutanweous coronary revascularization. *New England Journal of Medicine*, 2000, *342*, 1316-24.

[52] Symphony investigators. Comparison of sibrafiban with aspirin for prevention of cardiovascular events after acute coronary syndromes: a randomized trial. *Lancet*, 2000, *355*, 337-45.

[53] Quinn, M.J., Fitzgerald, D.J. Ticlopidine and clopidogrel. *Circulation*, 1999, *100*, 1667-72.

[54] Topol, E.J. *Heart*, 2000, *83*, 122-6.

[55] Gresele, P., Angnelli, G. Novel approaches to the treatment of thrombosis. *Trends in Pharmacological Sciences*, 2002, *23*, 25-32.

[56] Savage, B., Almus-Jacobs, F., Ruggeri, Z. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*, 1998, *94*, 657-666.

[57] de Groot, P.G., Ottenhof-Rovers, M., van Mourik, J.A., Sixma, J.J. Evidence that the primary binding site of von Willebrand factor that mediates platelet adhesion on subendothelium is not collagen. *J. Clin. Invest.*, 1988, *82(1)*, 65-73.

[58] Ruggeri, Z.M., Dent, J.A., Saldivar, E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood*, 1999, *94(1)*, 172-8.

[59] Granlick, H.R., Williams, S., McKeown, L., Kramer, W., Krutzxch, H., Gorecki, M., Pinet, A., Garfinkel, L.I. A monomeric von Willebrand factor fragment Leu 504-Lys728 inhibits von Willebrand factor interaction with glycoprotein Ib-IX. *Proceedings of the National Academy of Sciences*, 1992, *89*, 7880-4.

[60] Gurevitz, O., Goldfarb, A., Hod, H., Feldman, M., Shenkman, B., Varon, D., Eldar, M., Inbal, A. Recombinant von Willebrand factor fragment AR545C inhibits platelet aggregation and enhances thrombolysis with rtPA in a rabbit thrombosis model. *Arteriosclerosis Thrombosis and Vascular Biology*, 1998, *18*, 200-7.

[61] Cauwenberghs, N., Meiring, M., Vauterin, S., van Wyk, V., Lamprecht, S., Roodt, J.P., Novak, L., Harsfalvi, J., Deckmyn, H., Kotze, H.F. Antithrombotic effect of platelet glycoprotein Ib-blocking monoclonal antibody Fab fragments in non-human primates. *Arteriosclerosis Thrombosis and Vascular Biology*, 2000, *20*, 1347-53.

[62] Cook, J.J., Sitko, G.R., Bednar, B., Condra, C., Mellott, M.J., Feng, D.M., Nutt, R.F., Shafer, J.A., Gould, R.J., Connolly, T.M. An antibody against exosite of the cloned thrombin receptor inhibits experimental arterial thrombosis in the african green monkey. *Circulation*, 1995, *91*, 2961-71.

[63] Hasan, A.A.K., Rebello, S.S., Smith, E., Srikanth, S., Werns, S., Driscoll, E., Faul, J., Brenner, D., Normalle, D., Lucchesi, B.R., Schmaier, A.H. Thrombostatin inhibits induced canine coronary thrombosis. *Thrombosis and Haemostasis*, 1999, *82*, 1182-7.

[64] Gachet, A. ADP receptors and their inhibition. *Thrombosis and Haemostasis*, 2001, *86*, 222-32.

[65] Leon, C., Freund, M., Ravanat, C., Baurand, A., Cazenave, J.P., Gachet, C. Key role of the P2Y1 receptor in tissue factor-induced thrombin-dependent acute thromboembolism: studies in P2Y1 knockout mice and mice treated with P2Y1 antagonist. *Circulation*, 2001, *103*, 718-23.

[66] Hirsh, J. New anticoagulants. *American Heart Journal*, 2001, *142*, S3-S8.

[67] Broze, G.J. Jr. The role of tissue factor pathway inhibitor in a revised coagulation cascade. *Semin Hematol.*, 1992, *29*, 159-69.

[68] Erikkson, B.I., Wille-Jorgensen, P., Kalebo, P., Mouret, P., Rosencher, N., Bosch, P., Baur, M., Ekman, S., Bach, D., Lindbratt, S., Close, P. A comparison of recombinant hirudin with a low molecular-weight heparin to prevent thromboembolic complications after total hip replacement. *New England Journal of Medicine*, 1997, *337*, 1329-35.

[69] van der Werf, F. New data in treatment of acute coronary syndromes. *American Heart Journal*, 2001, *142*, S16-21.

[70] Direct thrombin inhibitor trialists' collaborative group Direct thrombin inhibitors in acute coronary syndromes: principal results of a meta-analysis based on individual patients' data. *Lancet*, 2002, *359*, 294-302.

[71] Weitz, J.I., Crowther. M. Direct thrombin inhibitors. *Thrombosis Research*, 2002, *106*, V275-84.

[72] Sporn, L.A., Marder, V.J., Wagner, D.D. von Willebrand factor released from Weibel-Palade bodies binds more avidly to extracellular matrix than that secreted constitutively. *Blood*, 1987, *69(5)*, 1531-4.

[73] Ruggeri, Z. Old concepts and new developments in the study of platelet aggregation. *J. Clin. Invest.*, 2000, *105*, 699-701.

[74] Ruggeri, Z. von Willebrand factor. *J. Clin. Invest.*, 1997, *110(11 Suppl)*, S41-6.

[75] Diacovo, T.G., Puri, K.D., Warnock, R.A., Springer, T.A., von Andrian, U.H. Platelet-mediated lymphocyte delivery to high endothelial venules *Science*, 1996, *273(5272)*, 252-5.

[76] Theilmeier, G., Lenaerts, T., Remacle, C., Collen, D., Vermylen, J., Hoylaerts, M.F. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. *Blood*, 1999, *94(8)*, 2725-34.

[77] Methia, N., Andre, P., Denis, C.V., Economopoulos, M., Wagner, D.D. Localized reduction of atherosclerosis in von Willebrand factor-deficient mice. *Blood*, 2001, *98(5)*, 1424-8.

[78] Theilmeier, G., Michiels, C., Spaepen, E., Vreys, I., Collen, D., Vermylen, J., Hoylaerts, M. Endothelial von Willebrand factor recruits platelets to atherosclerosis-prone sites in response to hypercholesterolemia. *Blood*, 2002, *99(12)*, 4486-4493.

[79] Furlan, M. Von Willebrand factor: molecular size and functional activity. *Ann. Hematol.*, 1996, *72(6)*, 341-8.

[80] Meyer, D., Obert, B., Pietu, G., Lavergne, J.M., Zimmerman, T.S. Multimeric structure of factor VIII/von Willebrand factor in von Willebrand's disease. *J. Lab Clin. Med.*, 1980, *95(4)*, 590-602.

[81] Doucet-de Bruine, M.H., Sixma, J.J., Over, J., Beeser-Visser, N.H. Heterogeneity of human factor VIII. II. Characterization of forms of factor VIII binding to platelets in the presence of ristocetin. *J. Lab Clin. Med.*, 1978, *92(1)*, 96-107.

[82] Ruggeri, Z.M., Zimmerman, T.S. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J. Clin. Invest.*, 1980, *65(6)*, 1318-25.

[83] Fujikawa, K., Suzuki, H., McMullen, B., Chung, D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood*, 2001, *98(6)*, 1662-6.

[84] Zheng, X., Chung, D., Takayama, T.K., Majerus, E.M., Sadler, J.E., Fujikawa, K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J. Biol. Chem.*, 2001, *276(44)*, 41059-63.

[85] Levy, G.G., Nichols, W.C., Lian, E.C., Foroud, T., McClintick, J.N., McGee, B.M., Yang, A.Y., Siemieniak, D.R., Stark, K.R., Gruppo, R., Sarode, R., Shurin, S.B., Chandrasekaran, V., Stabler, S.P., Sabio, H., Bouhassira, E.E., Upshaw, J.D., Jr., Ginsburg, D., Tsai, H.M. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*, 2001, *413(6855)*, 488-94.

[86] Xie, L., Chesterman, C.N., Hogg, P.J. Reduction of von Willebrand factor by endothelial cells. *Thromb. Haemost.*, 2000, *84(3)*, 506-13.

[87] Xie, L., Chesterman, C.N., Hogg, P.J. Control of von Willebrand factor multimer size by thrombospondin-1. *J. Exp. Med.*, 2001, *193(12)*, 1341-9.

[88] Pimanda, J.E., Annis, D.S., Raftery, M., Mosher, D.F., Chesterman, C.N., Hogg, P.J. The von Willebrand Factor reducing activity of thrombospondin-1 is located in the calcium-binding/C-terminal sequence and requires a free thiol at position 974. *Blood*, 2002, *100*(6), 2832-38

[89] Second International Study of Infarct Survival Collaborative Group. Randomized trial of intravenous streptokinase, oral aspirin, both or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. *Lancet*, 1988, *2*, 349-60.

[90] Topol, E.J., Bell, W.R., Weisfeldt, M.L. Coronary thrombolysis with recombinant tissue plasminogen activator in atherosclerotic thrombotic occlusion. *Journal of the American College of Cardiology*, 1985, *5*, 85-91.

[91] Carney, R.J., Murphy, G.A., Brandt, T.R., Daley, P.J., Pickering, C., White, H.J., McDonagh, T.J., Vermilya, S.K., Teichman, S.L. Randomized angiographic trial of recombinant tissue-type plasminogen activator (alteplase) in myocardial infarction. *Journal of the American College of Cardiology*, 1992, *20*, 17-23.

[92] The GUSTO investigators. An international randomized trial of comparing four thrombolytic strategies for acute myocardial infarction. *New England Journal of Medicine*, 1993, *329*, 673-82.

[93] The GUSTO investigators. An international, multicenter, randomized comparison of reteplase with alteplase for acute myocardial infarction. *New England Journal of Medicine*, 1997, *337*, 1118-23.

[94] Bode, C., Smalling, R.W., Berg, G., Burnett, C., Lorch, G., Kalbfleisch, J.M., Chernoff, R., Christie, L.G., Feldman, R.L., Seals, A.A., Weaver, W.D., for the RAPID investigators. Randomized comparison fo coronary thrombolysis achieved with double-bolus reteplase (recombinant tissue plasminogen activator) in patients with acute myocardial infarction. *Circulation*, 1996, *94*, 891-8.

[95] Verstraete, M. Third-generation thrombolytic drugs. *American Journal of Medicine*, 2000, *109*, 52-58.

[96] ASSENT-2 investigators. Single-bolus tenecteplase compared with front-loaded alteplase in acute myocardial infarction: the ASSENT-2 double blind randomised trial. *Lancet*, 1999, *354*, 716-22.

[97] Laroche, Y., Heymans, S., Capaert, S., de Cock, F., Demarsin, E., Collen, D. Recombinant staphylokinase variants with reduced antigenicity due to elimination of B-lymphocyte epitopes. *Blood*, 2000, *96*, 1425-32.

[98] Sinnaeve, P., van de Werf, F. Thrombolytic therapy. State of the art. *Thrombosis research*, 2001, *103*, S71-S79.

[99] Witt, W., Maass, B., Baldus, B., Hildebrand, M., Donner, P., Scheuning, W.D. Coronary thrombolysis with Desmodus salivary plasminogen activator in dogs. Fast and persistent recanalization by intravenous bolus administration. *Circulation*, 1994, *90*, 421-6.

[100] Montoney, M, Gardel, S.J., Marder, V.J. Comparison of the bleeding potential of vampire bat salivary plasminogen acitvator versus tissue plasminogen activator in an experimental rabbit model. *Circulation*, 1995, *91*, 1540-44.

[101] Topol, E.J., Yadev,J.S. Recognition of the importance of embolization in atherosclerotic vascular disease. *Circulation*, 2000, *101*, 570-80.

[102] Lincoff, A.M. GUSTO IV; expanding therapeutic options in acute coronary syndromes. *American Heart Journal*, 2000, *140*, S103-14.

[103] Ronner, E., van Kesteren, H.A., Zijnen, P., Altmann, E., Molhock, P.G., van der Wieken, L.R., Cuffie-Jackson, C.A., Neuhaus, K.L., Simmons, M.L. Safety and efficacy of eptifibatide vs placebo in patients receiving thrombolytic therapy with streptokinase for acute myocardial infarction; a phase II dose escalation, randomized, double-blind study. *European Heart Journal*, 2000, *21*, 1530-6.

[104] Verheugt, F.W. Risk and benefit of half-dose lytic pls abciximab versus lytic alone for ST-elevation myocardial infarction: a meta-analysis. *Journal of the American College of Cardiology*, 2002, *39(Suppl A)*, 828-6A.

[105] Antman, E.M., Louwerenburg, H.W., Baars, H.F., Wesdorp, J.G., Hamer, B., Bassand, J.P., Bigonzi, F., Pisapia, G., Gibson, C.M., Heidbuchel, H., Braunwald, E.,van der Werf, F. Enoxaparin as adjunctive antithrombin therapy for ST-elevation myocardial infarction: results of the ENTIRE-Thrombolysis in Myocardial Infarction (TIMI) 23 Trial. *Circulation*, 2002, *105*, 1642-9.

[106] Sleight P. Angiotensin II and Trials of Cardiovascular Outcomes. *American Journal of Cardiology*, 2002, *89*, 11A-17A.

[107] Cohn, J.N., Tognoni, G. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. *New England Journal of Medicine*, 2001, *345*, 1667-75.

[108] Yusuf, S. From the HOPE to the ONTARGET and the TRANSCEND studies: challenges in improving prognosis. *American Journal of Cardiology*, 2002, *89*, 18A-26A.

[109] Schmitz-Spanke, S., Schipke, J.S. Potential role of endothelin-1 and endothelin antagonists in cardiovascular diseases. *Basic Research in Cardiology*, 2000, *95*, 290-8.

[110] Karne, S., Jayawickreme, C.K., Lerner, M.R. Cloning and characterization of an endothelin-3 specific receptor (ETc receptor) for Xenopus laevis dermal melanopores.

Journal of Biological Chemistry, 1993, *268*, 18126-19133.

[111] Khurana, R., Martin, J.F., Zachary, I. Gene therapy for cardiovascular disease. A case for cautious optimism. *Hypertension*, 2001, *38*, 1210-16.

[112] Lowe, H.C., Oesterle, S.N., Burkhoff, D. Alternatives to traditional coronary bypass surgery. *Semin Thorac. Cardiovasc. Surg.*, 2002, *14*, 110-8.

[113] Kocher, A.A., Schuster, M.D., Szabocs, M.J., Takuma, S., Burkhoff, D., Wang, J., Homma, S., Edwards, N.M., Itescu, S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nature Medicine*, 2001, *7*, 430-6.

[114] Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., Isner, J.M. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*, 1997, *275*, 964-967.

[115] Ito, W.D., Arras, M., Scholz, D., Winkler, B., Htun, P., Schaper, W. Angiogenesis but not collateral growth is associated with ischemia after femoral artery occlusion. *American Journal of Physiology*, 1997, *273*(3 Pt 2), 111255-1265.

[116] Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine*, 2000, *6*, 389-95.

[117] Isner, J.M. Myocardial gene therapy. *Nature*, 2002, *415*, 234-9.

[118] Epstein, S.E., Fuchs, S., Zhou, Y.F., Baffour, R., Kornowski, R. Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. *Cardiovascular Research*, 2001, *49*, 532-42.

[119] Vale, P.R., Losordo, D.W., Milliken, C.E., Maysky, M., Esakof, D.D., Symes, J.F., Isner, J.M. Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. *Circulation*, 2000, *102*, 965-74.

[120] Dehmer, G.J. In *Textbook of Interventional Cardiology*, Topol. EJ, Ed.; WB Saunders, Philadelphia, 1994, Vol. 1, pp. 112-137.

[121] Gotto, A.M. Statin therapy: where are we? Where do we go next? *American Journal of Cardiology*, 2001, *87*, 13B-18B.

[122] McTaggert, F., Buckett, L., Davidson, R., Holdgate, G., McCormick, A., Schneck, D., Smith, G., Warwick, M. Preclinical and clinical pharmacology of rosuvastatin, a new 3-hydroxy-3-mehtylglutaryl coenzyme A reductase inhibitor. *American Journal of cardiology*, 2001, *87* (suppl.), 28B-32B.

[123] Olsson, A.G. Statin therapy and reductions in low-density lipoprotein cholesterol: initial clinical data on the potent new statin rosuvastatin. *American Journal of Cardiology*, 2001, *87*, 33B-36B.

[124] Brown, W.V. Novel approaches to lipid lowering: what is on the horizon? *American Journal of Cardiology*, 2001, *87*, 23B-27B.

[125] Lipka, L.J., LeBeaut, A.P., Veltri, E.P., Mellars, L.E., Bays, H.L., Moore, P.B. Reduction of LDL-cholesterol and elevation of HDL-cholesterol in subjects with primary hypercholesterolemia by SCH 58235: pooled analysis of two phase II studies. *Journal of the American College of Cardiology*, 2000, *35*, 257A.

[126] Benoit, P., Emmanuel, F, Caillaud, J.M., Bassinet, L., Castro, G., Galliz, P., Fruchart, J.C., Branellec, D., Denefle, P., Duverger, N. Somatic gene transfer of human apoA-I inhibits atherosclerosis progression in mouse models. *Circulation*, 1999, *99*, 105-110.

[127] Brenner, M.K., Pinkel, D. Cure of leukemia. *Semin Hematol.*, 1999, *36*(4 Suppl. 7), 73-83.

[128] Pinkel, D. Five-year follow-up of childhood lymphocytic leukemia. *JAMA*, 1971, *216*(4), 648-52.

[129] Ermolaeva, O., Rastogi, M., Pruitt, K.D., Schuler, G.D., Bittner, M.L., Chen, Y., Simon, R., Meltzer, P., Trent, J.M., Boguski, M.S. Data management and analysis for gene expression arrays. *Nat. Genet.*, 1998, *20*(1), 19-23.

[130] Alizadeh, A.A., Eisen, M.B., Davis, R.E., Ma, C., Lossos, I.S., Rosenwald, A., Boldrick, J.C., Sabet, H., Tran, T., Yu, X., Powell, J.I., Yang, L., Marti, G.E., Moore, T., Hudson, J., Jr., Lu, L., Lewis, D.B., Tibshirani, R., Sherlock, G., Chan, W.C., Greiner, T.C., Weisenburger, D.D., Armitage, J.O., Warnke, R., Staudt, L.M. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*, 2000, *403*(6769), 503-11.

[131] Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Lander, E.S. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*, 1999, *286*(5439), 531-7.

[132] Scherf, U., Ross, D.T., Waltham, M., Smith, L.H., Lee, J.K., Tanabe, L., Kohn, K.W., Reinhold, W.C., Myers, T.G., Andrews, D.T., Scudiero, D.A., Eisen, M.B., Sausville, E.A., Pommier, Y., Botstein, D., Brown, P.O., Weinstein, J.N. A gene expression database for the molecular pharmacology of cancer. *Nat. Genet.*, 2000, *24*(3), 236-44.

[133] Druker, B.J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G.M., Fanning, S., Zimmermann, J., Lydon, N.B. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med.*, 1996, *2*(5), 561-6.

[134] Monaco, R., Chen, J.M., Friedman, F.K., Brandt-Rauf, P., Chung, D., Pincus, M.R. Structural effects of the binding of GTP to the wild-type and oncogenic forms of the ras-gene-encoded p21 proteins. *J. Protein Chem.*, 1995, *14*(8), 721-9.

[135] Karp, J.E., Lancet, J.E., Kaufmann, S.H., End, D.W., Wright, J.J., Bol, K., Hawk, I., Tidwell, M.L., Liesveld, J., Kottke, T.J., Ange, D., Buddharaju, L., Gojo, I., Highsmith, W.E., Belly, R.T., Hohl, R.J., Rybak, M.E., Thibault, A., Rosenblatt, J. Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase 1 clinical-laboratory correlative trial. *Blood*, 2001, *97*(11), 3361-9.

[136] Frank, D.A. STAT signaling in the pathogenesis and treatment of cancer. *Mol. Med.*, 1999, *5*(7), 432-56.

[137] Marra, G., Chang, C.L., Laghi, L.A., Chauhan, D.P., Young, D., Boland, C.R. Expression of human MutS homolog 2 (hMSH2) protein in resting and proliferating cells. *Oncogene*, 1996, *13*(10), 2189-96.

[138] Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I., Seiser, C., Lazar, M.A., Minucci, S., Pelicci, P.G. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature*, 1998, *391*(6669), 815-8.

[139] He, L.Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., Pandolfi, P.P. Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat. Genet.*, 1998, *18*(2), 126-35.

[140] Lin, R.J., Nagy, L., Inoue, S., Shao, W., Miller, W.H., Jr., Evans, R.M. Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature*, 1998, *391*(6669), 811-4.

[141] Golub, T.R., Barker, G.F., Bohlander, S.K., Hiebert, S.W., Ward, D.C., Bray-Ward, P., Morgan, E., Raimondi, S.C., Rowley, J.D., Gilliland, D.G. Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. *Proc. Natl. Acad. Sci. USA*, 1995, *92*(11), 4917-21.

[142] Liu, P., Tarle, S.A., Hajra, A., Claxton, D.F., Marlton, P., Freedman, M., Siciliano, M.J., Collins, F.S. Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. *Science*, 1993, *261*(5124), 1041-4.

[143] Elledge, S.J. Cell cycle checkpoints: preventing an identity crisis. *Science*, 1996, *274*(5293), 1664-72.

[144] Levine, A.J. p53, the cellular gatekeeper for growth and division. *Cell*, 1997, *88*(3), 323-31.

[145] Reed, J.C. Double identity for proteins of the Bcl-2 family. *Nature*, 1997, *387*(6635), 773-6.

[146] Beg, A.A., Baltimore, D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*, 1996, *274*(5288), 782-4.

[147] Webb, A., Cunningham, D., Cotter, F., Clarke, P.A., di Stefano, F., Ross, P., Corbo, M., Dziewanowska, Z. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet*, 1997, *349*(9059), 1137-41.

[148] Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., Pehamberger, H. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet*, 2000, *356*(9243), 1728-33.

[149] Trapani, J.A., Smyth, M.J. Functional significance of the perforin/granzyme cell death pathway. *Nat. Rev. Immunol.*, 2002, *2*(10), 735-47.

[150] Raja, S.M., Wang, B., Dantuluri, M., Desai, U.R., Demeler, B., Spiegel, K., Metkar, S.S., Froelich, C.J. Cytotoxic cell granule-mediated apoptosis. Characterization of the macromolecular complex of granzyme B with serglycin. *J. Biol. Chem.*, 2002, *277*(51), 49523-49530.

[151] Trosko, J.E., Ruch, R.J. Gap junctions as targets for cancer chemoprevention and chemotherapy. *Curr. Drug Targets*. 2002, *3*(6), 465-82.

[152] Adams, J., Palombella, V.J., Sausville, E.A., Johnson, J., Destree, A., Lazarus, D.D., Maas, J., Pien, C.S., Prakash, S., Elliott, P.J. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res.*, 1999, *59*(11), 2615-22.

[153] Chandra, J., Niemer, I., Gilbreath, J., Kliche, K.O., Andreeff, M., Freireich, E.J., Keating, M., McConkey, D.J. Proteasome inhibitors induce apoptosis in glucocorticoid-resistant chronic lymphocytic leukemic lymphocytes. *Blood*, 1998, *92*(11), 4220-9.

[154] Miller, R.A., Maloney, D.G., Warnke, R., Levy, R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N. Engl. J. Med.*, 1982, *306*(9), 517-22.

[155] Sievers, E.L., Appelbaum, F.R., Spielberger, R.T., Forman, S.J., Flowers, D., Smith, F.O., Shannon-Dorcy, K., Berger, M.S., Bernstein, I.D. Selective ablation of acute myeloid leukemia using antibody-targeted chemotherapy: a phase I study of an anti-CD33 calicheamicin immunoconjugate. *Blood*, 1999, *93*(11), 3678-84.

[156] Kaminski, M.S., Estes, J., Zasadny, K.R., Francis, I.R., Ross, C.W., Tuck, M., Regan, D., Fisher, S., Gutierrez, J., Kroll, S., Stagg, R., Tidmarsh, G., Wahl, R.L. Radioimmunotherapy with iodine (131)I tositumomab for relapsed or refractory B-cell non-Hodgkin lymphoma: updated results and long-term follow-up of the University of Michigan experience. *Blood*, 2000, *96*(4), 1259-66.

[157] Hsu, F.J., Benike, C., Fagnoni, F., Liles, T.M., Czerwinski, D., Taidi, B., Engleman, E.G., Levy, R. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.*, 1996, *2*(1), 52-8.

[158] Reichardt, V.L., Okada, C.Y., Stockerl-Goldstein, K.E., Bogen, B., Levy, R. Rationale for adjuvant idiotypic vaccination after high-dose therapy for multiple myeloma. *Biol. Blood Marrow Transplant.*, 1997, *3*(3), 157-63.

[159] Hart, D.N. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood*, 1997, *90*(9), 3245-87.

[160] Nair, S.K., Heiser, A., Boczkowski, D., Majumdar, A., Naoe, M., Lebkowski, J.S., Vieweg, J., Gilboa, E. Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. *Nat. Med.*, 2000, *6*(9), 1011-7.

[161] Lum, L.G., LeFever, A.V., Treisman, J.S., Garlic, N.K., Hanson, J.P., Jr. Immune modulation in cancer patients after adoptive transfer of anti-CD3/anti-CD28-costimulated T cells-phase I clinical trial. *J. Immunother.*, 2001, *24*(5), 408-19.

[162] Alyea, E.P., Soiffer, R.J., Canning, C., Neuberg, D., Schlossman, R., Pickett, C., Collins, H., Wang, Y., Anderson, K.C., Ritz, J. Toxicity and efficacy of defined doses of CD4(−) donor lymphocytes for treatment of relapse after allogeneic bone marrow transplant. *Blood*, 1998, *91*(10), 3671-80.

[163] Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C., Le Douarin, N.M. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc. Natl. Acad. Sci. USA*, 1997, *94*(10), 5141-6.

[164] Gunsilius, E., Duba, H.C., Petzer, A.L., Kahler, C.M., Grunewald, K., Stockhammer, G., Gabl, C., Dirnhofer, S., Clausen, J., Gastl, G. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet*, 2000, *355*(9216), 1688-91.

[165] Risau, W. Mechanisms of angiogenesis. *Nature*, 1997, *386*(6626), 671-4.

[166] Habeck, M. Australian scientists design novel antiangiogenic weapon. *Lancet Oncology*, 2000, *1*, 199.

[167] Folkman, J. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N. Engl. J. Med.*, 1995, *333*(26), 1757-63.

[168] Feldman, A.L., Libutti, S.K. Progress in antiangiogenic gene therapy of cancer. *Cancer*, 2000, *89*(6), 1181-94.

[169] Gordon, M.S., Margolin, K., Talpaz, M., Sledge, G.W., Jr., Holmgren, E., Benjamin, R., Stalter, S., Shak, S., Adelman, D. Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. *J Clin Oncol*, 2001, *19*(3), 843-50.

[170] D'Amato, R.J., Loughnan, M.S., Flynn, E., Folkman, J. Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci. USA*, 1994, *91*(9), 4082-5.

[171] Smolich, B.D., Yuen, H.A., West, K.A., Giles, F.J., Albitar, M., Cherrington, J.M. The antiangiogenic protein kinase inhibitors SU5416 and SU6668 inhibit the SCF receptor (c-kit) in a human myeloid leukemia cell line and in acute myeloid leukemia blasts. *Blood*, 2001, *97*(5), 1413-21.

[172] Rochlitz, C.F. Gene therapy of cancer. *Swiss Med. Wkly*, 2001, *131*(1-2), 4-9.

[173] Roth, J.A., Nguyen, D., Lawrence, D.D., Kemp, B.L., Carrasco, C.H., Ferson, D.Z., Hong, W.K., Komaki, R., Lee, J.J., Nesbitt, J.C., Pisters, K.M., Putnam, J.B., Schea, R., Shin, D.M., Walsh, G.L., Dolormente, M.M., Han, C.I., Martin, F.D., Yen, N., Xu, K., Stephens, L.C., McDonnell, T.J., Mukhopadhyay, T., Cai, D. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nat. Med.*, **1996**, *2*(9), 985-91.

[174] Till, J.E., McCulloch, E.A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Res.*, **1961**, *14*, 213-222.

[175] Metcalf, D., Nicola, N.A. The hemopoietic Colony Stimulating Factors. From Biology to Clinical Applications., Cambridge University Press: Cambridge, **1994**.

[176] Metcalf, D. Cellular hematopoiesis in the twentieth century. *Semin Hematol.*, **1999**, *36*(4 Suppl. 7), 5-12.

[177] Cazzola, M., Messinger, D., Battistel, V., Bron, D., Cimino, R., Enller-Ziegler, L., Essers, U., Greil, R., Grossi, A., Jager, G., et al. Recombinant human erythropoietin in the anemia associated with multiple myeloma or non-Hodgkin's lymphoma: dose finding and identification of predictors of response. *Blood*, **1995**, *86*(12), 4446-53.

[178] Negrin, R.S., Stein, R., Vardiman, J., Doherty, K., Cornwell, J., Krantz, S., Greenberg, P.L. Treatment of the anemia of myelodysplastic syndromes using recombinant human granulocyte colony-stimulating factor in combination with erythropoietin. *Blood*, **1993**, *82*(3), 737-43.

[179] Hellstrom-Lindberg, E., Ahlgren, T., Beguin, Y., Carlsson, M., Carneskog, J., Dahl, I.M., Dybedal, I., Grimfors, G., Kanter-Lewensohn, L., Linder, O., Luthman, M., Lofvenberg, E., Nilsson-Ehle, H., Samuelsson, J., Tangen, J.M., Winqvist, I., Oberg, G., Osterborg, A., Ost, A. Treatment of anemia in myelodysplastic syndromes with granulocyte colony-stimulating factor plus erythropoietin: results from a randomized phase II study and long-term follow-up of 71 patients. *Blood*, **1998**, *92*(1), 68-75.

[180] Mantovani, L., Lentini, G., Hentschel, B., Wickramanayake, P.D., Loeffler, M., Diehl, V., Tesch, H. Treatment of anaemia in myelodysplastic syndromes with prolonged administration of recombinant human granulocyte colony-stimulating factor and erythropoietin. *Br. J. Haematol.*, **2000**, *109*(2), 367-75.

[181] Hellstrom-Lindberg, E. Efficacy of erythropoietin in the myelodysplastic syndromes: a meta-analysis of 205 patients from 17 studies. *Br. J. Haematol.*, **1995**, *89*(1), 67-71.

[182] Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N. Engl. J. Med.*, **2002**, *346*(7),469-75.

[183] Ohno, R. Granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor in the treatment of acute myeloid leukemia and acute lymphoblastic leukemia. *Leuk. Res.*, **1998**, *22(12)*, 1143-54.

[184] Kaushansky, K. Thrombopoietin: from theory to reality. *Thromb. Haemost.*, **1999**, *82(2)*, 312-7.

[185] Basser, R.L., O'Flaherty, E., Green, M., Edmonds, M., Nichol, J., Menchaca, D.M., Cohen, B., Begley, C.G. Development of pancytopenia with neutralizing antibodies to thrombopoietin after multicycle chemotherapy supported by megakaryocyte growth and development factor. *Blood*, **2002**, *99(7)*, 2599-602.

[186] Broudy, V.C. Stem cell factor and hematopoiesis. *Blood*, **1997**, *90(4)*, 1345-64.

[187] Biwa, T., Sakai, M., Shichiri, M., Horiuchi, S. Granulocyte/macrophage colony-stimulating factor plays an essential role in oxidized low density lipoprotein-induced macrophage proliferation. *J. Atheroscler. Thromb.* **2000**, *7*, 14-20.

[188] High, K.A. AAV-mediated gene transfer for hemophilia. *Ann. N. Y. Acad. Sci.*, **2001**, *953*, 64-74.

[189] Weatherall, D.J., Clegg, J.B. Genetic disorders of hemoglobin. *Semin Hematol.*, **1999**, *36(*4 Suppl. 7*)*, 24-37.

[190] Franchini, M., Gandini, G., de Gironcoli, M., Vassanelli, A., Borgna-Pignatti, C., Aprili, G. Safety and efficacy of subcutaneous bolus injection of deferoxamine in adult patients with iron overload. *Blood*, **2000**, *95*(9), 2776-9.

[191] Cohen, A.R., Galanello, R., Piga, A., Dipalma, A., Vullo, C., Tricta, F. Safety profile of the oral iron chelator deferiprone: a multicentre study. *Br. J. Haematol.*, **2000**, *108*(2), 305-12.

[192] Colman, A., Kind, A. Therapeutic cloning: concepts and practicalities. *Trends Biotechnol.*, **2000**, *18(5)*, 192-6.

[193] Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J.P., Thrasher, A.J., Wulffraat, N., Sorensen, R., Dupuis-Girod, S., Fischer, A., Davies, E.G., Kuis, W., Leiva, L., Cavazzana-Calvo, M. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med.*, **2002**, *346*(16), 1185-93.

[194] Marshall, E. Clinical research. Gene therapy a suspect in leukemia-like disease. *Science*, **2002**, *298(5591)*, 34-5.

[195] Hughes, S. Cardiac stem cells. *Journal of Pathology*, **2002**, *197*, 468-78.

Inhibition of Experimental Neointimal Hyperplasia and Thrombosis Depends on the Type of Vascular Injury and the Site of Drug Administration

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Background. Heparin inhibits vascular smooth muscle cell proliferation in tissue culture and limits neointimal hyperplasia after experimental arterial injury but has been ineffective in reducing clinical restenosis. We examined how this discrepancy might reflect suboptimal drug-tissue interactions and/or differences in the vascular response to injury.

Methods and Results. Intravenous infusion was compared with local administration of heparin to injured rabbit iliac arteries either from drug-impregnated polymeric controlled release matrices in the perivascular space or from drug-releasing endovascular stents. Occlusive thrombosis, seen in 42% of control stent-bearing arteries, and partial thrombosis were virtually eliminated by heparin delivery from any route. Intimal area 14 days after balloon withdrawal denudation alone was reduced to an equal extent by continuous systemic heparin or by perivascular heparin for the first 3 days. In contrast, endovascular stents produced more exuberant neointimal hyperplasia, the inhibition of which required continuous rather than only early heparin administration. Neither perivascular delivery limited to the first 3 days nor stent-based delivery reduced neointimal hyperplasia as effectively.

Conclusions. The antiproliferative and antithrombotic effects of heparin differ markedly, depending on the type of arterial injury and the mode of drug administration. Different forms of injury may require different therapies, and complications of arterial intervention such as excessive neointimal hyperplasia and thrombosis may demand alternate therapeutic regimens. Duration, dose, and site of delivery rather than frank resistance to therapy may explain why experimentally effective antiproliferative and antithrombotic agents fail clinically. (*Circulation.* 1993;88:1215-1221.)

KEY WORDS • restenosis • stents • heparin

Revascularization of obstructive atherosclerotic vessels induces thrombosis and neointimal hyperplasia, which in turn cause recurrent luminal narrowing. This process is so severe as to require additional intervention in 30% to 40% of coronary arteries after balloon angioplasty and in over 60% of aortocoronary saphenous vein bypass grafts within 5 years after surgery.[1-3] The lesions in these diseases are composed primarily of vascular smooth muscle cells (SMC) and extracellular matrix, and efforts to limit neointimal hyperplasia have focused on a diverse set of compounds that inhibit SMC growth and thrombosis in tissue culture and in animal models of acute arterial injury.[4-10] With most compounds, however, translation of experimental success to clinical use aimed at inhibiting restenosis has failed.[11-21]

Heparin, independent of anticoagulant properties, inhibits SMC proliferation in tissue culture[5,22] as well as in denuded rat carotid arteries[4] whether administered systemically or, in much lower doses, to the adventitial surface.[23] In contrast, heparin delivered to the luminal aspect of experimentally damaged arteries has reduced neither neointimal hyperplasia nor thrombosis,[24-26] and clinical trials using systemic heparin after balloon angioplasty have failed to reduce clinical or angiographic restenosis.[14,27,28] Although thrombosis has been proposed as a direct contributor to intimal hyperplasia after experimental endovascular stent placement,[29] elimination of thrombosis by heparin has not reduced the rate of restenosis after coronary stent placement.[30,31]

We now report that the means of drug administration, site of drug delivery, and extent of arterial injury all contribute to the biological response to heparin. Neointimal hyperplasia and thrombosis were followed after single acute injury (balloon withdrawal denudation) or more chronic and severe injury (balloon withdrawal followed by endovascular stent placement) in rabbit iliac arteries. Arteries were treated with heparin delivered via local intra-arterial, local perivascular, or systemic intravenous routes. Although all forms of heparin treatment reduced stent thrombosis and limited neointimal hyperplasia after balloon withdrawal injury, only continuous perivascular or intravenous drug delivery produced effective inhibition of neointimal hyperplasia

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after the more chronic and severe injury of endovascular stents. These results may help explain the numerous clinical failures of experimentally effective regimens aimed at limiting restenosis.

Methods

Animal Care and Surgical Procedure

Twenty-five New Zealand White rabbits (Millbrook Farm Breeding Labs, Amherst, Mass) of either sex, weighing 3.5 to 4 kg, were housed in individual mesh cages and maintained on rabbit chow and water. Beginning 1 day before surgery, aspirin (0.07 mg/mL, Sigma, St Louis, Mo) was added to drinking water for an approximate daily dose of 5 mg/kg.

Anesthesia was achieved with an intramuscular injection of ketamine (35 mg/kg, Aveco Co, Fort Dodge, Iowa), followed by intravenous sodium Nembutal (Abbott Laboratories, North Chicago, Ill) 4 mg/kg via a marginal ear vein. All animals received a single intravenous bolus of heparin (100 U/kg, Elkins-Sinn Inc, Cherry Hill, NJ) at the time of surgery. Animals were maintained on a warming blanket throughout surgery and recovery. Both femoral arteries were exposed and ligated, and catheters passed via arteriotomy. The endothelium of the iliac arteries was denuded with a 3F balloon embolectomy catheter (Baxter Healthcare Corp, Edwards Division, Santa Ana, Calif), inflated in the abdominal aorta, and withdrawn three times to the femoral artery. Six animals received no further arterial manipulation (balloon group). In another group of 19 animals, a stainless steel slotted-tube stent, 7 mm in length, was inflated within each iliac artery (stent group). Each stent was mounted on a 3-mm angioplasty balloon (Advanced Cardiovascular Systems Inc, Santa Clara, Calif) and expanded with a steady 15-second inflation at 10 atm pressure. The arteries had approximate diameters of 2.5 mm, for a balloon stent to artery ratio of 1.2:1.

Heparin Administration

Ethylene-vinyl acetate copolymer (EVAc) matrices, 33% loaded with heparin (Choay heparin 1453, 12 000 to 18 000 DA, USP 160 U/mg, Paris), were prepared as previously described.[23] Matrices (10×5×1 mm) were covered with either two or six coats of EVAc, and two 20-gauge or 27-gauge holes were bored at equal spacings into one matrix face. In this manner, heparin release was constrained to provide desired release kinetics. Heparin release was measured in vitro by incubating either heparin-impregnated EVAc matrices or stents with ionically bound heparin in lactated Ringer's solution at 37°C for 16 days. Aliquots of solution were sampled at regular intervals, and their heparin content was assayed using the metachromasia of Azure A (Fisher Scientific Co, Fairlawn, NJ) at 620 nm.[32] Because heparin release from EVAc matrices was either highest after 2 days of incubation or was approaching steady-state release, (depending on the number of EVAc coats and the size of the holes), matrices were incubated at 37°C in lactated Ringer's solution for 48 hours before implantation in vivo.

In addition to the intraoperative bolus of heparin and daily oral aspirin, experimental groups received one of the following: no additional treatment (12 arteries in the stent group, 4 arteries in the balloon group); continuous left femoral intravenous infusion of heparin 0.3 $mg \cdot kg^{-1} \cdot h^{-1}$ via an osmotic pump (Alza Corp, Palo Alto, Calif) (4 arteries in the stent group, 4 arteries in the balloon group); controlled release of heparin from an EVAc matrix placed under the inguinal ligament and positioned in the perivascular space directly adjacent to the balloon-injured arteries (4) or stent-bearing arteries (10); or elution of heparin from an intra-arterial metal stent (12 arteries). Activated partial thromboplastin times (aPTT) were measured using a desktop analyzer (Ciba-Corning Diagnostics Corp, Oberlin, Ohio) at the time of procedure, 7 days later, and at the time the animals were killed.

Tissue Processing

Arteries were harvested 14 days after surgery. After a lethal intravenous injection of sodium Nembutal, inferior vena caval exsanguination, and 100 mm Hg pressure infusion of lactated Ringer's solution via left ventricular puncture, the iliac arteries were excised and fixed in Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid).

Nonstented arterial segments were embedded in paraffin and cut in 6-μm cross sections. Stent-bearing arterial segments were isolated, oriented for proximal and distal ends, and embedded in K-Plast (Medim America Ltd, Wilmington, Del). Four to eight 5-μm arterial cross sections were then cut with a tungsten carbide knife from three sites along each stent: proximal end, middle, and distal end. This allowed integration of histological observations over the entire length of each stent, minimizing sampling error. Metal stent sections were not removed from the arteries before embedding. All sections were stained with Verhoeff's tissue elastin stain.

Intimal cross-sectional area was determined by means of computer-assisted digital planimetry. The extent of deep arterial injury caused by stent wires was quantified using the method of Schwartz et al.[33] The antithrombotic efficacy of various heparin regimens was compared by contrasting both the frequency of complete stent thrombosis as well as the percent of the cross-sectional circumference of patent stent-bearing arteries covered with laminar thrombus (measured on histological sections with computer-aided planimetry).

Statistics

All data are presented as the mean±SE. Statistical analysis comparing treatment groups used a nonpaired *t* test. Values of $P<.05$ were considered significant.

Results

Heparin Release Rates

Ethylene-vinyl acetate copolymer matrix slabs were constructed to release heparin with either rapid first order or more prolonged near-zero order kinetics (Fig 1). Matrix rectangles with two copolymer coats and two 20-gauge holes released heparin in a first-order manner with a peak rate of 50.6±4.6 μg/h after 60 hours' incubation. Because all matrices were incubated for 48 hours before in vivo implantation, this first matrix formulation provided peak heparin release 12 hours after insertion into experimental animals, with little

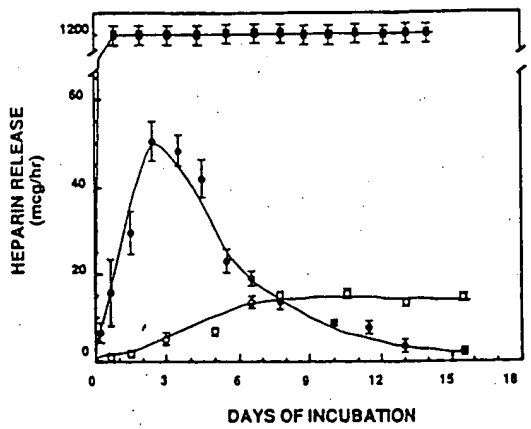

FIG 1. *Graph shows heparin release rates in vitro in lactated Ringer's solution at 37°C from osmotic minipumps (closed squares) or polymeric heparin-impregnated matrices (open and closed circles). Matrices were constructed to release heparin primarily early during incubation (closed circles) or in a continuous fashion over 14 days (open circles). Each method was subsequently applied for heparin delivery to rabbit iliac arteries in vivo, pumps for intravenous infusion, and polymeric matrices for controlled perivascular delivery. Before in vivo use, pumps were incubated in lactated Ringer's solution at 37°C for 6 hours and matrices for 2 days.*

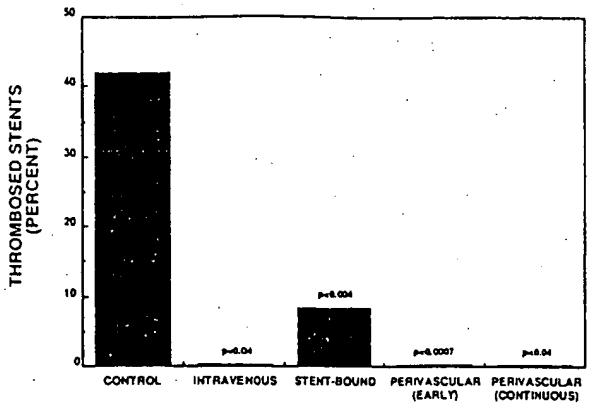

FIG 2. *Bar graph shows incidence of complete luminal thrombosis in rabbit iliac arteries 14 days after balloon withdrawal denudation followed by endovascular stent placement. Treated groups received heparin via intravenous infusion, from the stent itself, or from drug-impregnated matrices placed adjacent to the adventitia of stent-bearing arteries. Matrices were incubated in vitro for 2 days before placement and provided heparin release either early (ie, primarily for the first 3 days after placement) or continuously over 14 days. Probability values reflect comparison with untreated controls.*

release after the third in vivo day. More prolonged-release kinetics were obtained by applying six copolymer coats and constraining release to two smaller 27-gauge holes. This matrix formulation released heparin more gradually, reaching near-zero order kinetics (heparin release rates were between 13.4±1.4 and 15.2±0.6 μg/h) after the first 5 days. Again, because of 48 hours of preincubation before in vivo use, this second matrix formulation provided an increasing heparin release rate for the first 3 in vivo days, with steady-state release thereafter. Ionically bound heparin was released from metal stents at a rate of 1.0 μg/h for the first day, rapidly declining to a steady-state release rate of 0.3±0.1 μg/h for the ensuing days. Osmotic pumps delivered heparin at $300\pm6\ \mu g \cdot kg^{-1} \cdot h^{-1}$ (Fig 1), as determined by manufacturer's specifications and confirmed by determination of pump residual volumes at the completion of experiments.

Anticoagulation and Thrombosis

Activated partial thromboplastin times were measured in animals before surgery and at 7 and 14 days. Continuous intravenous heparin infusion prolonged the aPTT to at least two times control in each animal at 7 and 14 days. There was no prolongation of the aPTT by either perivascular or stent-released heparin in any animal at any time point.

Complete thrombosis at 14 days was observed in 42% of the arteries in animals receiving a single intravenous bolus of heparin at the time of stent placement and oral aspirin continuously (see Table and Fig 2). All forms of heparin administration reduced thrombosis in stent-bearing arteries. Only 8% of arteries implanted with stents releasing heparin demonstrated complete thrombosis ($P<.004$), whereas heparin delivered either intravenously or into the perivascular space completely eliminated occlusive thrombosis (see Table and Fig 2). As a measure of partial thrombosis, the percent of arterial circumference covered with laminar thrombus on histo-

Antithrombotic and Antiproliferative Effects of Different Modes of Heparin Delivery on Rabbit Iliac Arteries 14 Days After Vascular Injury

	Intima (mm^2)			Thrombosis	
Heparin	Balloon	Stent	Injury score	Complete	Partial
None	0.26±0.03	1.15±0.11	0.56±0.07	42%	29±6%
Intravenous	0.12±0.03	0.41±0.05	0.54±0.02	0%	0±0%
	($P<.03$)	($P<.002$)		($P<.04$)	($P<.04$)
Perivascular	0.11±0.03	0.82±0.08	0.50±0.08	0%	14±5%
(early)	($P<.03$)	($P<.03$)		($P<.0007$)	($P<.03$)
Perivascular		0.52±0.08	0.55±0.14	0%	12±4%
(continuous)		($P<.006$)		($P<.04$)	($P<.04$)
Stent-based		1.09±0.11	0.63±0.07	8%	17±4%
		(P=NS)		($P<.004$)	($P<.05$)

Injury was caused by balloon withdrawal denudation alone (balloon) or with accompanying endovascular stent placement (stent). Probability values reflect comparison with control animals receiving no postprocedure heparin.

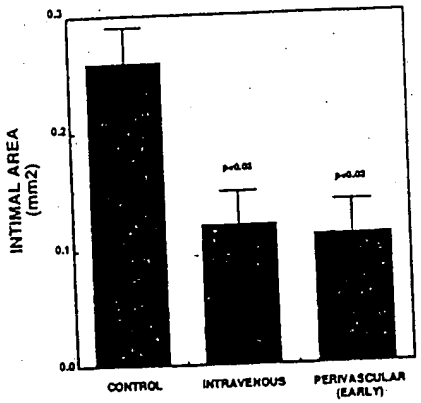

FIG 3. *Bar graph shows cross-sectional intimal area 14 days after balloon withdrawal denudation of rabbit iliac arteries. Treated groups received heparin either via continuous intravenous infusion or from drug-impregnated matrices placed adjacent to the adventitia of injured arteries providing heparin release primarily for the first 3 days after placement. Probability values reflect comparison with untreated controls.*

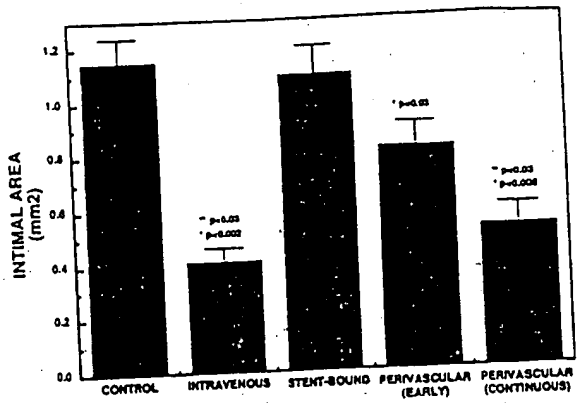

FIG 4. *Bar graph shows cross-sectional intimal area 14 days after balloon withdrawal denudation followed by endovascular stent placement in rabbit iliac arteries. Treated groups received heparin either via intravenous infusion or from the stent itself or from matrices placed adjacent to the adventitia of stent-bearing arteries. Matrices were incubated in vitro for 2 days before placement and provided heparin delivery either early (ie, primarily for the first 3 days after placement) or continuously over 14 days. *Compared with untreated controls; **compared with early perivascular delivery.*

logical cross section was calculated for each group. All modes of heparin delivery reduced partial mural thrombosis: stent-released heparin reduced partial thrombosis from 29±6% to 17±4%; intravenous heparin infusion eliminated partial thrombosis altogether (0±0%); and early or continuous perivascular heparin reduced partial thrombosis to 14±5% or 12±4%, respectively ($P<.04$ for each heparin-treated group compared with control, P=NS between heparin-treated groups; see Table).

Neointimal Hyperplasia

We compared heparin modulation of the arterial response to acute injury (balloon withdrawal with endothelial denudation) with the effect of heparin on the more chronic and severe injury imposed by the placement of metal stents within denuded arteries. Fourteen days after balloon injury alone, a highly cellular neointima had formed, separating the internal elastic lamina from the lumen. Intimal cross-sectional area was 0.26±0.03 mm² (see Table and Fig 3). Heparin after balloon injury was equally effective at inhibiting neointimal hyperplasia whether delivered via continuous intravenous infusion for 14 days or via controlled perivascular release from EVAc matrices providing local delivery of much lower doses primarily for the first 3 days (see Table and Fig 3; intimal areas 0.12±0.03 mm² or 0.11±0.03 mm², respectively; $P<.03$ for each compared with controls). The degree of inhibition of neointimal hyperplasia, expressed as the percent reduction in intravenously or locally treated groups compared with untreated controls, was 54% or 57%, respectively. Furthermore, balloon withdrawal alone resulted in no disruption of the internal elastic lamina.

The placement of an endovascular metal stent induced an intimal response more than fourfold greater than balloon withdrawal alone (see Table and Figs 4 and 5A; intimal area, 1.15±0.11 mm²). Heparin was delivered by way of intravenous osmotic pump infusion, intra-arterial heparin-bound stent implants, or heparin-impregnated polymeric matrices deployed in the perivascular space. Intimal area after intravenous delivery was 0.41±0.05 mm², a 64% reduction compared with stented controls ($P<.002$; see Table and Figs 4 and 5B). Stent-based release of heparin had no significant effect on intimal area (1.09±0.11 mm²; see Table and Figs 4 and 5C). Perivascular heparin delivered primarily early after stent placement with little delivery after the third day reduced intimal area 29% to 0.82±0.08 mm² ($P<.03$ compared with controls; $P<.02$ compared with intravenous heparin group; see Table and Figs 4 and 5D), whereas more prolonged continuous local perivascular heparin release achieved an almost twofold greater reduction of 54% in intimal area (0.52±0.08 mm²; $P<.006$ compared with stented controls and $P<.03$ compared with early perivascular heparin treatment, respectively; see Table and Figs 4 and 5E). Stent-based heparin release was ineffective, and perivascular heparin delivery for only 3 days after stent placement less effective than continuous intravenous or perivascular delivery at reducing intimal hyperplasia, despite near complete elimination of stent thrombosis.

Quantification of the extent of deep vessel injury[33] revealed an injury score of 0.56±0.07 in arterial sections from control animals receiving stents but not postprocedure heparin. There was no significant difference between the control group and any heparin-treated group (Table).

Discussion

Heparin inhibited thrombosis regardless of the method of delivery and neointimal hyperplasia irrespective of the type of arterial injury, but the reduction in the intimal response after more severe and chronic injury elicited by endovascular stents was attained only with prolonged heparin delivery continuing for the duration of the experiment. Small doses of heparin released into the perivascular space primarily for the first 3 days after stent placement or at the arterial lumen reduced thrombosis but had little effect on neointimal hyperplasia, whereas heparin delivered via continuous

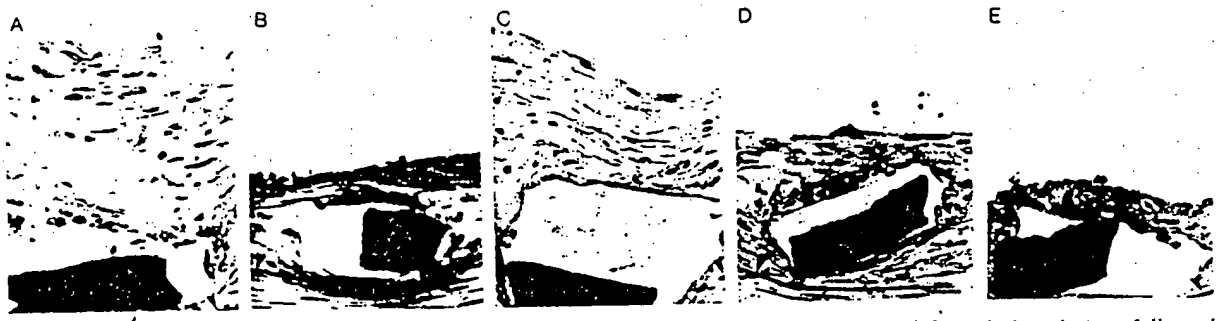

FIG 5. *Photomicrographs of rabbit iliac arterial cross sections 14 days after balloon withdrawal denudation followed by endovascular stent placement. A, Control; B, receiving continuous intravenous heparin; C, receiving heparin released from the stent itself; D, receiving heparin delivered into the perivascular space for the first 3 days after injury; or E, receiving heparin delivered into the perivascular space continuously for 14 days. Neointimal hyperplasia separates the stent wires (black rectangles) and internal elastic laminae (arrows) from the lumina. Open space around wires is an artifact of sectioning. Original magnification ×400; stained with Verhoeff's tissue elastin stain.*

intravenous infusion or continuous local perivascular release reduced stent-induced neointimal hyperplasia by 64% and 54%, respectively.

In arteries subjected to a single superficial acute injury (balloon withdrawal denudation), deep mural injury was less common and neointimal hyperplasia was fourfold less extensive than after stent placement. Local perivascular delivery of heparin primarily over the first 3 days after single acute vascular injury was as effective as continuous intravenous heparin for 14 days (reducing neointimal area by 54% and 57%, respectively). These data confirm an earlier report that after rat carotid artery denudation, 3 or 7 days of intravenous heparin reduced SMC growth fraction, migration, and accumulation as effectively as more prolonged administration.[34] In contrast, our data show that after endovascular stent placement, 3 days of local heparin delivery was markedly less effective than either continuous intravenous dosing or continuous local perivascular release at limiting neointimal growth.

Stent Thrombosis and Intimal Hyperplasia

Clinical coronary arterial stent placement has been complicated by both early thrombosis and subsequent neointimal hyperplasia leading to restenosis.[30,31,35-37] Extremely potent antithrombotic regimens including antiplatelet, anticoagulant, and thrombolytic agents have been used to reduce the rate of clinically recognized acute thrombosis from 24% to 39%[30,37] to less than 3%.[31,36] Not unexpectedly, these regimens have been associated with a high incidence of vascular complications occurring in up to 16% of patients.[36]

A direct link has been proposed between thrombosis and neointimal hyperplasia induced by stents in swine coronary arteries,[29] based on the observation that systemic administration of anticoagulant heparin reduced neointimal hyperplasia after stent placement in a similar model.[38] In carotid or coronary arteries of swine treated with aspirin, however, heparin released from metal stents neither significantly reduced thrombosis nor limited intimal response, although the incidence of thrombosis in control animals was low.[24,25] Moreover, although heparin in conjunction with other antiplatelet and anticoagulant drugs after human coronary stenting has virtually eliminated thrombosis, angiographic restenosis rates of 25% to 32% have persisted in clinical trials.[30,31,36] Our data show that although heparin regimens that curtailed thrombosis such as continuous intravenous or perivascular delivery also greatly limited neointimal hyperplasia, other regimens such as stent-released heparin or early postprocedure perivascular heparin reduced both partial and complete thrombosis at 14 days but had little to no effect on neointimal hyperplasia. The relation between these two aspects of stent-induced arterial injury remains to be fully delineated.

Type of Vascular Injury Dictates Antiproliferative Effects of Heparin

Endothelial denudation in experimental animals has long been used as a model of vascular response to injury. Elucidation of the cellular responses and growth regulation after such injury has allowed identification of diverse compounds capable of inhibiting neointimal hyperplasia after arterial damage.[4,6-10] Paradoxically, clinical investigations with these compounds after coronary interventions have failed to demonstrate beneficial reduction in restenosis.[11-21] Our data show that two different models of experimental vascular injury within the same animal arterial system respond quite differently to an agent with known and well-characterized antiproliferative activity.

Experimental endovascular stent placement is associated with more severe arterial damage and includes a more prolonged phase of intimal SMC proliferation than is seen after balloon withdrawal injury alone.[39,40] The number of proliferating SMC, identified by incorporation of bromodeoxyuridine or ^{3}H-thymidine, is highest in the first week after either balloon injury or stent placement and virtually ceases within 14 days of balloon denudation but continues at high levels for greater than 28 days after stent placement.[39-41] The fourfold increase in neointimal hyperplasia observed when endovascular stents were added to balloon-injured arteries may reflect a more extensive initial injury to the arterial wall[33] or a more chronic stimulus for proliferation related to the presence of the indwelling stent itself.

Others have also reported that local luminal heparin delivery via perforated perfusion balloons or from metal stents does not inhibit neointimal hyperplasia after experimental vascular injury.[24-26] Our data show that

local delivery of heparin to the adventitial surface but not to the luminal surface of stent-bearing arteries can reduce neointimal hyperplasia as effectively as systemic dosing. The failure of other methods of local heparin delivery may reflect differences in the biological activity of the heparin used, differences in the site of drug application (lumen vs adventitia), differences in the local concentrations of heparin achieved, or differences in the form of arterial injury (additional damage with a perfusion balloon vs single balloon injury vs primary stent deployment) rather than biological resistance to local heparin treatment. More chronic and severe arterial damage demands more prolonged administration of an antiproliferative agent to inhibit neointimal hyperplasia than does single denuding arterial injury.

The results in this study further highlight the divergence of human atherosclerosis and restenosis from animal models of acute arterial injury. The clinical failure of agents antiproliferative in some animal models may reflect differences in the extent of injury and/or differences between an otherwise normal, acutely injured blood vessel and an atherosclerotic vessel subjected to angioplasty or other manipulation. The transition from tissue culture and animal studies to human trials may be enhanced by further elucidation of the biological mechanisms that determine growth after severe or prolonged vascular insults.

Study Limitations

The site, duration, and amount of heparin delivered dictated the drug's modulation of thrombosis and neointimal hyperplasia after superficial acute or deeper chronic injury. We have not yet measured or localized the deposition of exogenous heparin within the arterial wall or correlated the distribution of drug with its biological effects. Measurement and localization of heparin in vivo are difficult because of heparin's high solubility and rapid degradation into smaller oligomers of varying biological activities. These issues are undergoing active investigation in our laboratory. The amount of heparin delivered from stents was small although adequate to reduce thrombosis: Stent-based heparin release provided only 2% the dose of heparin provided by perivascular matrix-based heparin delivery. The delivery of higher doses for longer periods of time might produce different results.

Future Directions

The accurate transition from experimental models of vascular injury to clinical reduction in accelerated arteriopathies may be limited by differences in the biology of animal models and human disease, differential responses of various components of vascular repair, and dissimilarities between human atherosclerotic arteries and normal vessels of the laboratory animal. We believe that an additional explanation may lie in the methods used to deliver agents and in the failure of clinical trials to adequately extrapolate experimental tissue culture and animal data to human use, rendering the broad application of negative conclusions from such clinical trials unwarranted. The extent of experimental neointimal hyperplasia and the response to an antiproliferative agent, heparin, differs significantly between arteries subjected to a single acute injury and arteries subjected to more prolonged and severe injury. The latter model may be more akin to clinical coronary arterial instrumentation, characterized by deep intimal and medial disruption. Future clinical studies of experimentally effective antiproliferative therapies will need to address duration and degree of drug-tissue interaction in light of the duration and degree of the vascular response to injury before such compounds are deemed ineffective at limiting human restenosis.

Acknowledgments

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References

1. Campeau L, Enjalbert M, Lesperance J, Bourassa MG, Kwiterovich P, Wacholder S, Sniderman A. The relation of risk factors to the development of atherosclerosis in saphenous-vein bypass grafts and the progression of disease in the native circulation. *N Engl J Med.* 1984;311:1329-1332.
2. Leimgruber PP, Roubin GS, Hollman J, Cotsonis GA, Meier B, Douglas JS Jr, King SB III, Gruentzig AR. Restenosis after successful coronary angioplasty in patients with single-vessel disease. *Circulation.* 1986;73:710-717.
3. Hirshfeld JW, Schwartz JS, Jugo R, Macdonald RG, Goldberg S, Savage MP, Bass TA, Vetrovec G, Cowley M, Taussig AS, Whitworth HB, Margolis JR, Hill JA, Pepine CJ. Restenosis after coronary angioplasty: a multivariate statistical model to relate lesion and procedure variables to restenosis. *J Am Coll Cardiol.* 1991;18:647-656.
4. Clowes AW, Karnovsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature.* 1977;265:625-626.
5. Hoover RL, Rosenberg RD, Haering W, Karnovsky MJ. Inhibition of rat arterial smooth muscle cell proliferation by heparin, II: in vitro studies. *Circ Res.* 1980;47:578-583.
6. Liu MW, Roubin GS, Robinson KA, Black AJR, Hearn JA, Siegel RJ, King SB III. Trapadil in preventing restenosis after balloon angioplasty in the atherosclerotic rabbit. *Circulation.* 1990;81:1089-1093.
7. Powell JS, Clozel J-P, Muller RKM, Kuhn H, Hefti F, Hosang M, Baumgartner HR. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science.* 1989;245:187-189.
8. Sarembock IJ, Gertz SD, Gimple LW, Owen RM, Powers ER, Roberts WC. Effectiveness of recombinant desulphatohirudin in reducing restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. *Circulation.* 1991;84:232-243.
9. Henry PD, Bentley KI. Suppression of atherogenesis in cholesterol-fed rabbits treated with nifedipine. *J Clin Invest.* 1981;68:1366-1369.
10. Jonasson L, Hol J, Hansson GK. Cyclosporin A inhibits smooth muscle proliferation in the vascular response to injury. *Proc Nad Acad Sci U S A.* 1988;85:2303-2306.
11. Serruys PW, Rutsch W, Heyndrickx GR, Danchin N, Mast G, Wijns W, Rensing BJ, Vos J, Stibbe J. Prevention of restenosis after percutaneous coronary angioplasty with thromboxane A2-receptor blockade. *Circulation.* 1991;84:1568-1580.
12. O'Keefe JH, McCallister BD, Bateman TM, Kuhnlein DL, Ligon RW, Hartzler GO. Ineffectiveness of colchicine for the prevention of restenosis after coronary angioplasty. *J Am Coll Cardiol.* 1992;19:1597-1600.
13. MERCATOR. Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? *Circulation.* 1992;86:100-110.
14. Ellis SG, Roubin GS, Wilentz J, Douglas JS Jr, King SB III. Effect of 18- to 24-hour heparin administration for prevention of restenosis after uncomplicated coronary angioplasty. *Am Heart J.* 1989;117:777-782.
15. Dehmer GJ, Pompa JJ, van den Berg EK, Eichhorn EJ, Prew[illegible]B, Campbell WB, Jennings L, Willerson JT, Schmitz JM. Rec[illegible]on

in the rate of early restenosis after coronary angioplasty by a diet supplemented with n-3 fatty acids. *N Engl J Med.* 1988;319:733-740.
16. Thornton MA, Gruentzig AR, Hollman J, King SB III, Douglas JS Jr. Coumadin and aspirin in the prevention of recurrence after transluminal coronary angioplasty: a randomized study. *Circulation.* 1984;69:721-727.
17. Schwartz L, Bourassa MG, Lesperance J, Aldridge HE, Kazim F, Salvatori VA, Henderson M, Bonan R, David PR. Aspirin and dipyridamole in the prevention of restenosis after percutaneous transluminal coronary angioplasty. *N Engl J Med.* 1988;318: 1714-1719.
18. Reis GJ, Sipperly ME, McCabe CH, Sacks FM, Boucher TM, Silverman DI, Baim DS, Grossman W, Pasternak RC. Randomized trial of fish oil for prevention of restenosis after coronary angioplasty. *Lancet.* 1989;2:177-181.
19. Whitworth HB, Roubin GS, Hollman J, Meier B, Leingruber PP, Douglas JS Jr, King SB III, Gruentzig AR. Effect of nifedipine in recurrent stenosis after percutaneous coronary angioplasty. *J Am Coll Cardiol.* 1986;8:1271-1276.
20. Pepine CJ, Hirshfeld JW, Macdonald RG, Henderson MA, Bass TA, Goldberg S, Savage MP, Vetrovec G, Cowley M, Taussig AS, Whitworth HB, Margolis JR, Hill JA, Bove AA, Jugo R. A controlled trial of corticosteroids to prevent restenosis after coronary angioplasty. *Circulation.* 1990;81:1753-1761.
21. Corcos T, David PR, Val PG, Renkin J, Dangiosse V, Rapold HG, Bourassa MG. Failure of diltiazem to prevent restenosis after percutaneous transluminal coronary angioplasty. *Am Heart J.* 1985;109:926-931.
22. Guyton JR, Rosenberg RD, Clowes AW, Karnovsky MJ. Inhibition of rat arterial smooth muscle cell proliferation by heparin: in vivo studies with anticoagulant and nonanticoagulant heparin. *Circ Res.* 1980;46:625-634.
23. Edelman ER, Adams DH, Karnovsky MJ. Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. *Proc Natl Acad Sci U S A.* 1990;87: 3773-3777.
24. Cavender JB, Anderson PA, Roubin GS. The effects of heparin bonded tantalum stents on thrombosis and neointimal proliferation. *Circulation.* 1990;82(suppl III):III-541. Abstract.
25. Cox DA, Anderson PG, Roubin GS, Chou CY, Agrawal SK, Cavender JB. Local delivery of heparin and methotrexate fails to inhibit in vivo smooth muscle cell proliferation. *Circulation.* 1991; 84(suppl II):II-71. Abstract.
26. Gimple LW, Gertz SD, Haber HL, Ragosta M, Powers ER, Roberts WC, Sarembock IJ. Effect of chronic subcutaneous or intramural administration of heparin on femoral artery restenosis after balloon angioplasty in hypercholesterolemic rabbits. *Circulation.* 1992;86:1536-1546.
27. Faxon D, Spiro T, Minor S, Douglas J, Cote G, Dorosti K, Gottlieb R, Califf R, Topol E, Gordon J. Enoxaprin, a low molecular weight heparin, in the prevention of restenosis after angioplasty: the results of a double blind randomized trial. *J Am Coll Cardiol.* 1992;19:258A. Abstract.
28. Lehmann KG, Doria RJ, Feuer JM, Hall PX, Hoang DT. Paradoxical increase in restenosis rate with chronic heparin use: final results of a randomized trial. *J Am Coll Cardiol.* 1991;17:181A. Abstract.
29. Schwartz RS, Holmes DH, Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. *J Am Coll Cardiol.* 1992;20:1284-1293.
30. Serruys PW, Strauss BH, Beatt KJ, Bertrand ME, Puel J, Rickards AF, Meier B, Goy J-J, Vogt P, Kappenberger L, Sigwart U. Angiographic follow-up of a self-expanding coronary-artery stent. *N Engl J Med.* 1991;324:13-17.
31. Ellis SG, Savage M, Fischman D, Baim DS, Leon M, Goldberg S, Hirshfeld JW, Cleman MW, Teirstein PS, Walker C, Bailey S, Buchbinder M, Topol EJ, Schatz RA. Restenosis after placement of Palmaz-Schatz stents in native coronary arteries. *Circulation.* 1992;86:1836-1844.
32. Gundry SR, Klein M, Drongowski RA, Kirsh MM. Clinical evaluation of a new rapid heparin assay using the dye Azure-A. *Ann Surg.* 1984;148:191-194.
33. Schwartz RS, Huber KC, Murphy JG, Edwards WD, Camrud AR, Vlietstra RE, Holmes DR. Restenosis and proportional neointimal response to coronary artery injury: results in a porcine model. *J Am Coll Cardiol.* 1992;19:267-274.
34. Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury, IV: heparin inhibits rat smooth muscle cell mitogenesis and migration. *Circ Res.* 1986;58:839-845.
35. Anderson PG, Bajaj RK, Baxley WA, Roubin GS. Vascular pathology of balloon-expandible flexible coil stents in humans. *J Am Coll Cardiol.* 1992;19:372-381.
36. Carrozza JP, Kuntz RE, Levine MJ, Pomerantz RM, Fishman RF, Mansour M, Gibson CM, Senerchia CC, Diver DJ, Safian RD, Baim DS. Angiographic and clinical outcome of intracoronary stenting: immediate and long-term results from a large single-center experience. *J Am Coll Cardiol.* 1992;20:328-337.
37. Puel J, Rousseau H, Joffre F, Hatem S, Fauvel JM, Bounhoure JP. Intravascular stents to prevent restenosis after transluminal coronary angioplasty. *Circulation.* 1987;76(suppl IV):IV-27. Abstract.
38. Buchwald AB, Unterberg C, Nebendahl K, Grone H-J, Wiegand V. Low-molecular-weight heparin reduces neointimal proliferation after coronary stent implantation in hypercholesterolemic minipigs. *Circulation.* 1992;86:531-537.
39. Hanke H, Hassenstein S, Kamenz J, Oberhoff M, Baumbach A, Betz E, Karsch KR. Prolonged proliferative response of smooth muscle cells after experimental intravascular stenting: a stent wire related phenomenon. *Circulation.* 1992;86(suppl I):I-186. Abstract.
40. Hanke H, Hassenstein S, Kamenz J, Oberhoff M, Baumbach A, Betz E, Karsch KR. Experimental intravascular stenting: proliferative response of smooth muscle cells. *Circulation.* 1991;84(suppl II):II-71. Abstract.
41. Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury, II: inhibition of smooth muscle cell growth by heparin. *Lab Invest.* 1985;52:611-616.

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THROMBOSIS RESEARCH

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Regular Article

The GP IIb/IIIa inhibitor abciximab (c7E3) inhibits the binding of various ligands to the leukocyte integrin Mac-1 (CD11b/CD18, $\alpha_M\beta_2$)

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Abstract

Cross-reactivity with integrins other than glycoprotein IIb/IIIa (GP IIb/IIIa) is discussed as a potential reason for the overall clinical benefits of the GP IIb/IIIa-blocking antibody-fragment abciximab. We evaluated whether abciximab binds to the leukocyte integrin Mac-1, whether it inhibits binding of the distinct ligands and thereby may modulate inflammation, cell proliferation and coagulation.

Binding of fluorescence-labelled abciximab to phorbolmyristate acetate-stimulated monocytes and to a monocytic cell line (THP-1) could be detected in flow cytometry. The binding of fibrinogen, the inactivated complement factor 3b (iC3b), and the coagulation factor X to Mac-1 could be inhibited by abciximab (10 μg/ml) in vitro. As a functional consequence, the conversion of factor X to factor Xa mediated by Mac-1, as detected by the chromogenic substrate SZ-2222, was impaired by abciximab. Adhesion of THP-1 cells to immobilized intercellular adhesion molecule 1 (ICAM-1) and to fibrinogen was reduced significantly by abciximab. Fibrinogen-mediated cell aggregation was also impaired.

In conclusion, we describe binding of abciximab to Mac-1 on stimulated monocytes. Thereby, abciximab inhibits binding of the ligands fibrinogen, ICAM-1, iC3b and factor X. Furthermore, we demonstrated that Mac-1-dependent conversion from factor X to factor Xa is impaired by abciximab, arguing for the direct modulation of the coagulation cascade by abciximab. Overall, the inhibition of Mac-1 could provide additional clinical benefits of abciximab beyond the well-described blockade of GP IIb/IIIa.

Keywords: Platelet aggregation inhibitors; Cell adhesion molecules; Leukocytes; Fibrinogen

1. Introduction

Adhesion molecules, particularly the integrin family, play a crucial role in vascular biology by mediating cell–cell and cell–matrix adhesion as well as by binding soluble ligands. The therapeutic blockade of the platelet integrin glycoprotein IIb/IIIa (GP IIb/IIIa) has evolved as a successful pharmacological approach in acute coronary syndromes and percutaneous coronary intervention. Various GP IIb/IIIa-blocking agents have been developed. The molecular basis of these blocking agents are either monoclonal antibodies (e.g. abciximab), ligand mimetic peptides (e.g. eptifibatide), or synthetic ligand mimetics (e.g. tirofiban) [1,2]. These agents vary in pharmacological properties such as receptor affinity and receptor specificity [3,4]. Until now, the most widely tested GP IIb/IIIa-blocking agent is the human/mouse chimeric monoclonal antibody (mAb) fragment abciximab. Reduction of mortality up to 60% has been reported and the overall long-term reduction of mortality in all abciximab trials is about 35% [5,6].

While the cross-inhibition of the vitronectin receptor ($\alpha_v\beta_3$) by abciximab is generally accepted [7], the inhibition of the leukocyte receptor Mac-1 ($\alpha_M\beta_2$) by abciximab and the potential role of this inhibition are still discussed controversially [8–12]. Altieri et al. [13] demonstrated binding of the original IgG of 7E3 to Mac-1 on a monocytic cell line using radioactive labelling and Simon et al. [14] demonstrated the binding of abciximab to Mac-1-expressing monocytic cells and the inhibition of adhesion to immobilized fibrinogen and intercellular adhesion molecule 1 (ICAM-1).

To further elucidate the potential effects of abciximab on the Mac-1 receptor, the binding of additional ligands, such as

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factor X and inactivated complement factor 3b (iC3b) and its inhibition by abciximab were investigated. Furthermore, we demonstrated modulation of factor X activation and thus modulation of the coagulation cascade by abciximab.

2. Materials and methods

2.1. Cells

The monocytic cell lines THP-1 and U937 were obtained from the tumor cell bank of the German Cancer Research Center (DKFZ, Heidelberg). The cells were maintained in RPMI 1640 medium, containing 10% heat-inactivated fetal calf serum, 2 mmol L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco).

2.2. Antibodies and reagents

The mouse/human chimeric Fab abciximab (c7E3) was purchased from Beiersdorf-Lilly. Abciximab was biotinylated with BAC-SulfoNHS (Sigma) at a pH of 7.2, resulting in a biotin/protein ratio of 1.6. The fluorescein isothiocyanate (FITC)-labelled polyclonal chicken anti-fibrinogen antibody was purchased from Biopool, the anti-CD11b (clone 44) mAb from Pharmingen, the anti-iC3b mAb from Biotrend and the secondary anti-Fab Ab from Immunotech. Anti-keyhole limpet mAb from Becton Dickinson was used as unspecific negative control.

Factor X (Sigma) was labelled with FITC (Pierce) at a pH of 9.3. Phorbol 12-myristate 13-acetate (PMA) for leukocyte stimulation, fibrinogen, *p*-nitrophenylphosphate and Triton X was purchased from Sigma, the chromogenic factor X substrate SZ-2222 from Chromo-genix and recombinant soluble ICAM-1 from R&D Systems.

2.3. Binding of abciximab, fibrinogen and factor X to monocytes

Blood was collected by venipuncture with a 21-gauge butterfly needle from healthy volunteers and anticoagulated with citric acid. Subsequently, incubation was performed with various concentrations of biotinylated abciximab, FITC-labelled polyclonal chicken anti-fibrinogen antibody or FITC-labelled factor X for 30 min at room temperature. If necessary, stimulation of cells was performed by the addition of 100 nM PMA. After the addition of 4 ml of "FACS-Lysing" solution (Becton Dickinson), centrifugation was performed with 1000 rpm (Sorvall 6000) for 10 minutes. The pellet was resuspended in 300 μl Cellfix (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). Monocytes were identified on the forward/sideward scatter.

2.4. Detection of Mac-1-mediated factor X conversion

Mac-1 expressing THP-1 cells were activated by addition of 100 nM PMA to the culture medium 12 h prior to starting the experiment. Cells were washed twice in modified Tyrode's buffer and then incubated for 30 min at room temperature with factor X (1 U/ml) in a 96-well plate, some in the presence of abciximab (20 μg/ml) or anti-Mac-1 mAb (20 μg/ml). Tyrode's buffer alone was used as negative control and factor Xa (1 U/ml) as positive control. Then, the chromogenic factor Xa substrate SZ-2222 (4 mM) was added. After another 10 min incubation at room temperature, the conver-

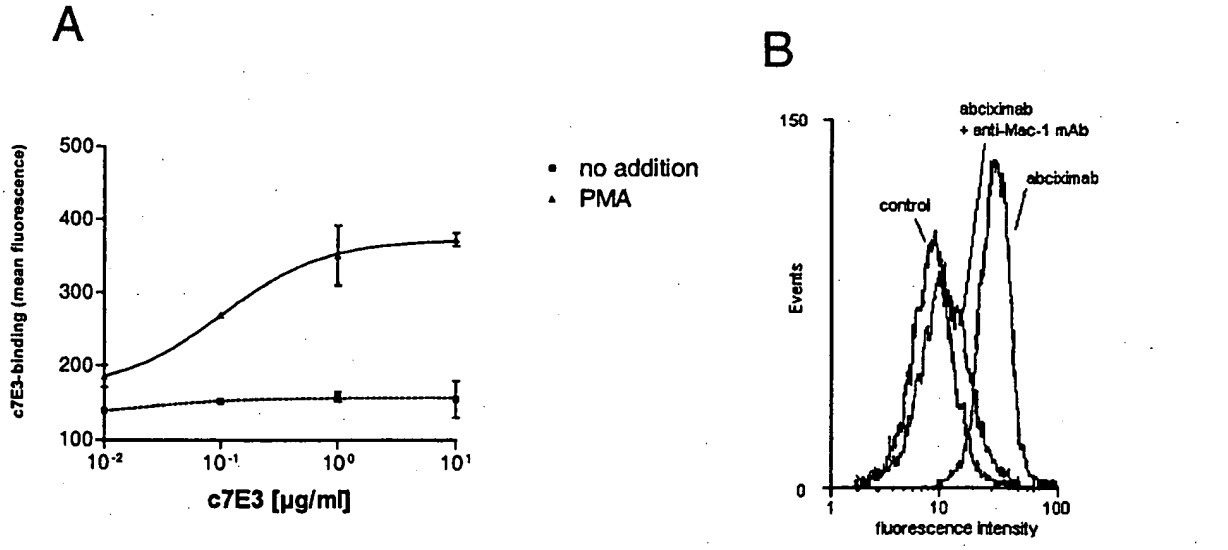

Fig. 1. Binding of abciximab to Mac-1. (A) Abciximab binding, as detected by flow cytometry with biotin-labelled abciximab to resting and PMA-stimulated monocytes. Depicted are mean fluorescence and standard error of the mean of three measurements. (B) Flow cytometry histogram of abciximab binding to PMA-activated monocytes, compared to the unspecific binding of a control antibody and after coincubation with a Mac-1-blocking mAb.

sion of the factor Xa substrate was measured in an ELISA-Reader (Multiscan, Titerteck) using a 405-nm filter.

2.5. iC3b binding to monocytes

Complement factor 3 (100 nM) was incubated overnight at room temperature. The conversion was started by addition of factor D (1 nM), factor B (5 nM) and 200 nM magnesium chloride and 30 min incubation at 37 °C. In a second step, factor H and factor I were added, and again, 30 min incubation at 37 °C was performed. Citrated blood was centrifuged at 1000 rpm for 10 min and the white fraction (buffy coat) was extracted. Cells were counted, diluted to 10000 leukocytes/ml and washed twice in modified Tyrode's buffer. After 20

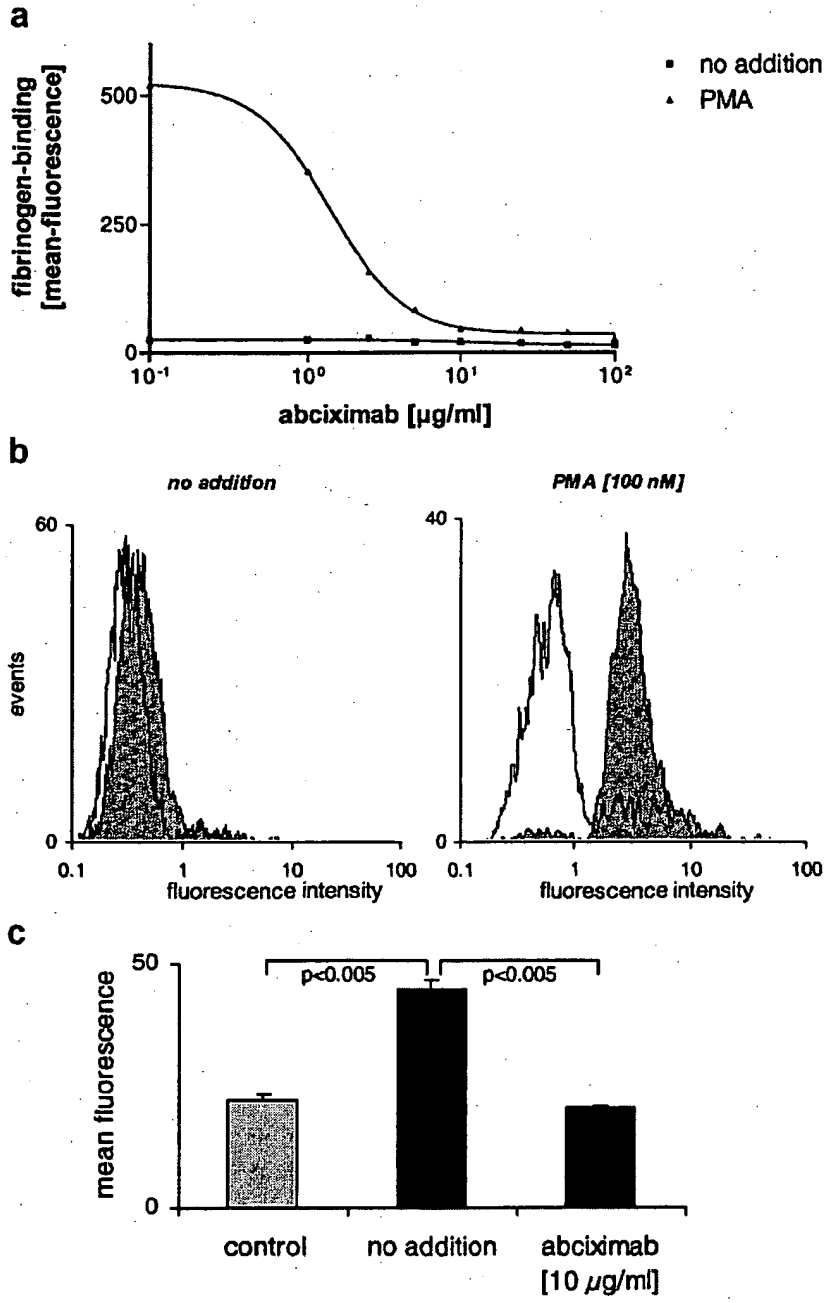

Fig. 2. Inhibition of ligand binding to Mac-1 by abciximab. (a) Fibrinogen binding to resting and activated blood-monocytes at increasing concentrations of abciximab, as measured in flow cytometry with a FITC-labelled polyclonal chicken anti-fibrinogen antibody. (b) Flow cytometry histograms of factor X binding to resting and PMA-activated monocytes. The cells, which were coincubated with abciximab (10 µg/ml), are depicted as white histograms, the cells without any addition as grey histograms. (c) iC3b binding to washed blood monocytes, as detected in flow cytometry using an anti-iC3b and a FITC-labelled anti-Fab-Fragment-specific antibody. Depicted are mean fluorescence and standard deviation of triplets.

min incubation with or without 10 μg/ml abciximab, 1 μl iC3 solution was added to 50 μl cell solution and incubated for 20 min. Lysis of remaining red blood cells and FACS (fluorescence-activated cell scan)-analysis was performed as described above.

2.6. Mac-1-mediated cell aggregation

U937 cells were stimulated with 100 nM PMA overnight, washed twice in Tyrode's buffer, counted and diluted to 100000 cells/μl. After incubation with abciximab (10 μg/ml) or no addition, fibrinogen solution to a final concentration of 3 g/l, or as negative control plain Tyrode's buffer, was added. Cells were then rotated 1 h at 100 rpm in a 6-well plate.

2.7. Adhesion of U937 cells to immobilized fibrinogen and ICAM-1

A 96-well plate was incubated overnight at 4 °C with fibrinogen (10 μg/ml) or recombinant ICAM-1 in PBS. After this, wells were washed with PBS and 10 mg/ml BSA in PBS was added to block unspecific binding sites. After 1 h incubation at 4 °C, the wells were washed twice with PBS. Then, 100000 cells per well in Tyrode's buffer, partially preincubated with 20 μg/ml abciximab or anti-Mac-1 mAb, were added and incubated 1 h at 37 °C. A 100-μl sample per well of Tyrode's buffer was then added and pipetted off twice cautiously to wash out nonadherent cells. To quantify the remaining adherent cells, the activity of the cell endogenous acid phosphatase was used by adding 100 μl of the following substrate/lysis solution to each well: 1% Triton X-100, 6 mg/ml *p*-nitrophenylphosphate in 50 mM sodium acetate buffer at pH 5. The reaction was terminated after 1 h incubation at 37 °C by addition of 50 μl NaOH (1 M). Then, conversion of the acid phosphatase substrate was measured using an ELISA plate reader (Multiscan, Titerteck) with a 405-nm filter.

3. Results

Abciximab binding to monocytes can be directly demonstrated by flow cytometry. Half-maximal binding of abciximab to activated monocytes was achieved at con-

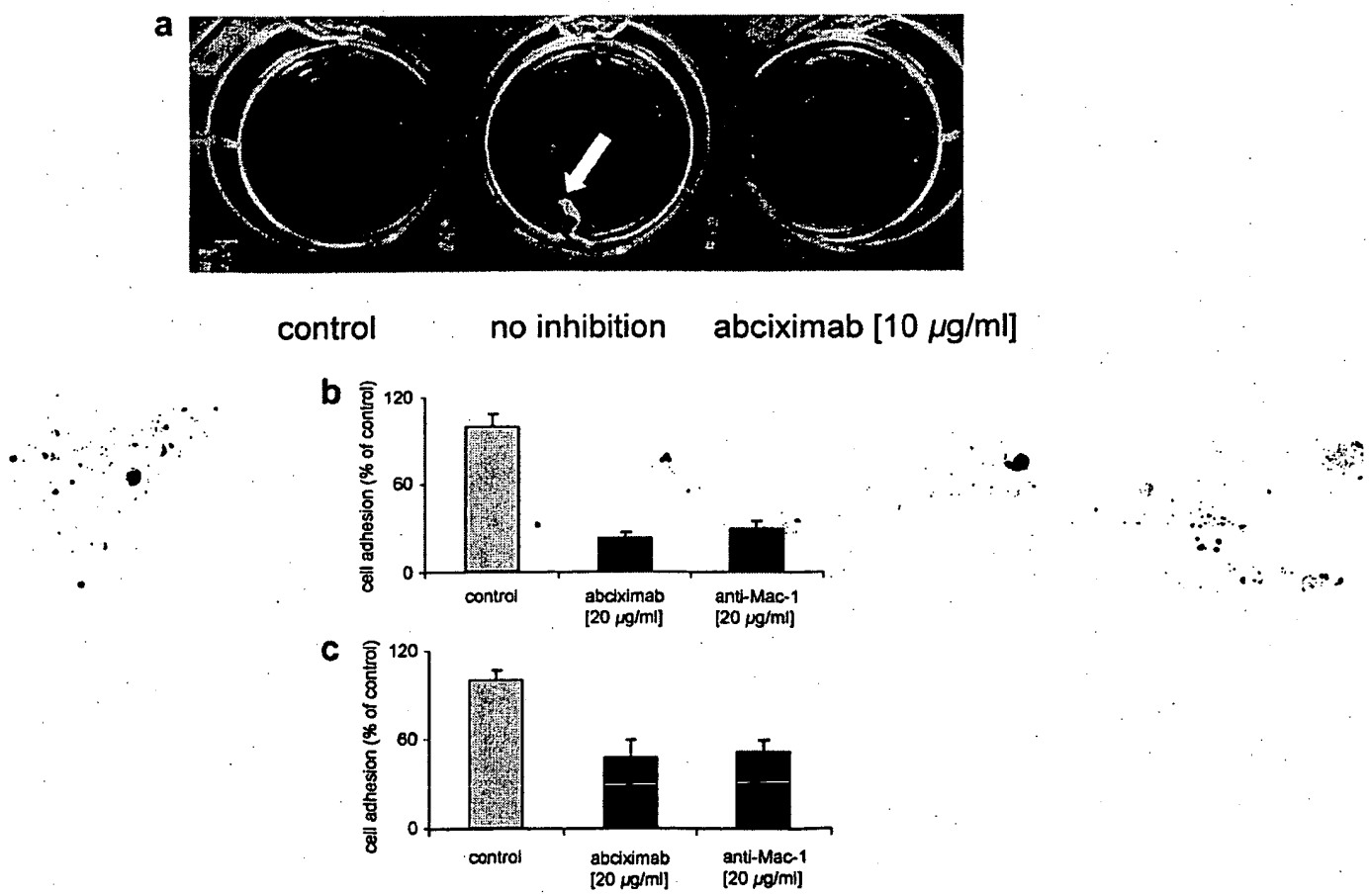

Fig. 3. (a) U937 cell aggregation in fibrinogen (3 g/l) solution. (b) U937 adhesion to immobilized fibrinogen, detected by the chromogenic endogenous acid phosphatase substrate *p*-nitrophenylphosphate. Depicted is the percentage of light absorption, in comparison to the control (mean value and standard deviation of three wells). (c) U937 adhesion to immobilized recombinant ICAM-1. Experimental procedures were performed as described in (b).

centrations between 0.1 and 1 μg/ml (Fig. 1A). Unstimulated monocytes did not bind abciximab. The specific binding to the adhesion molecule Mac-1 can be shown by the competition of binding to monocytes by a mAb that is specific for the α-subunit of Mac-1 (α_M). Preincubation with this Mac-1-specific mAb inhibits abciximab binding to monocytes (Fig. 1B). Thus, abciximab binds specifically to Mac-1 on monocytes. The same results were obtained with Mac-1-expressing THP-1 cells (not shown), thus arguing against platelet-contaminating effects in the monocyte preparations.

As a next step, we explored whether ligand binding to Mac-1 is influenced by abciximab. Monocytes were incubated with physiological concentrations of fibrinogen and with increasing concentrations of abciximab. Nonstimulated monocytes did not bind fibrinogen and the binding of fibrinogen to PMA-stimulated monocytes could be inhibited in a dose-dependent manner by abciximab (Fig. 2a). The binding of biotinylated factor X to blood monocytes could also be demonstrated in flow cytometry and was nearly totally inhibited by preincubation with abciximab (Fig. 2b). The complement factor iC3b was evaluated as third Mac-1 ligand. Binding of iC3b to monocytes that was inhibitable by a Mac-1-specific antibody could be demonstrated in flow cytometry (Fig. 2c).

The functional consequences of Mac-1 blockade were further evaluated. Fibrinogen is present in high concentration in human plasma and mediates cell aggregation based on its bivalent nature. We evaluated the ability of abciximab to inhibit fibrinogen-mediated monocyte aggregation. Monocytes were incubated with physiological fibrinogen concentrations on a rotator (100 rpm) for 1 h resulting in a large monocyte aggregate (Fig. 3a). Aggregation can be blocked by preincubation with 10 μg/ml abciximab (Fig. 3a). Furthermore, adhesion of monocytic THP-1 cells on immobilized fibrinogen was evaluated. Abciximab inhibits monocyte adhesion on fibrinogen to the same extent as an anti-Mac-1 mAb (Fig. 3b). In a similar experimental setting, adhesion of THP-1-cells to immobilized ICAM-1 was tested. As depicted in Fig. 3c, Mac-1-mediated adhesion to immobilized ICAM-1 is clearly impaired by abciximab.

To evaluate the consequence of the inhibition of factor X, Mac-1-mediated factor X activation was examined using a chromogenic factor Xa substrate. PMA-activated THP-1 cells facilitate the conversion of factor X to factor Xa. The specificity of this effect for Mac-1 could be proven by inhibition with a blocking anti-Mac-1 mAb. Blockade of Mac-1 with abciximab results in a significant inhibition of factor Xa-formation (Fig. 4), demonstrating an additional anticoagulative effect of abciximab.

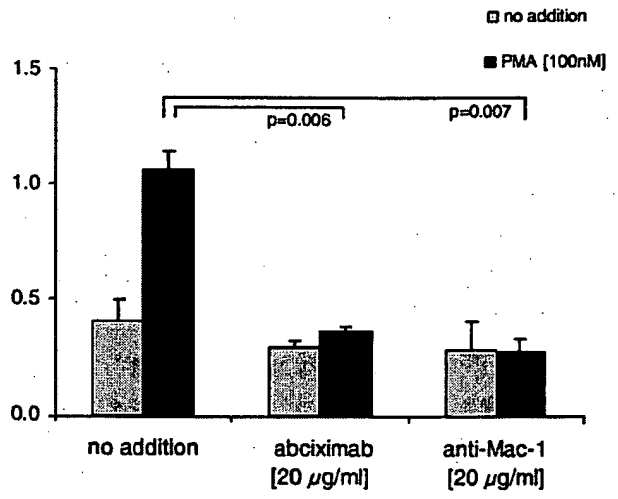

Fig. 4. Factor X conversion mediated by THP-1 cells, as measured by the chromogenic factor Xa substrate SZ-2222. Factor Xa activity was calculated as absorption ratio in comparison to the positive control (1 U/ml factor Xa). Results are expressed as mean and standard deviation of three measurements.

4. Discussion

The proven clinical benefits of abciximab are mainly attributed to the blockade of the platelet fibrinogen receptor GP IIb/IIIa. The ability of abciximab to cross-react with other integrin receptors such as the vitronectin or the Mac-1 receptor is discussed as an additional contribution to the clinical benefits of abciximab. The I-domain of the Mac-1 α-subunit has been proposed as the abciximab binding epitope on Mac-1 [15]. The I domain has also been proposed as the binding site for iC3b and various adhesive ligands of Mac-1 [16,17]. Therefore, we hypothesized that abciximab binding to Mac-1 might inhibit the binding of several ligands. Moreover, we examined the effects of abciximab on factor X binding to Mac-1 and the biological functions of Mac-1 such as cell adhesion and factor X activation.

The major findings of the present study are: (1) abciximab binds to the activated Mac-1 on monocytes and Mac-1-expressing THP-1 cells in vitro; (2) abciximab inhibits fibrinogen binding to Mac-1; (3) abciximab inhibits factor X binding to Mac-1 and Mac-1-mediated conversion of factor X to Xa; (4) abciximab inhibits fibrinogen-mediated monocyte aggregation and monocyte adhesion; (5) abciximab inhibits Mac-1-mediated monocyte adhesion on ICAM-1.

In the present study, we used ex vivo monocytes as well as monocytic cell lines expressing Mac-1. The use of ex vivo monocytes allowed an experimental setting very similar to the physiological situation, since lysis and fixation were performed not until stimulation and incubation with abciximab were completed. On the other hand, the use of the monocytic cell lines THP-1 and U937 allowed the exclusion of platelet contamination. These cells co-expressed other integrin receptors as $\alpha_v\beta_3$ only very weakly and specificity of abciximab binding to Mac-1 could be demonstrated by the inhibitory effects of a Mac-1-specific antibody. Even though the validity of in vitro studies is generally limited,

the combination of ex vivo and in vitro experiments demonstrates solid evidence for a direct Mac-1-blocking effect of abciximab. However, the clinical relevance of the effects described in this study still has to be confirmed.

What are the biological functions that could be affected by the blockade of Mac-1? The leukocyte integrin Mac-1 is expressed on monocytes and granulocytes and has, as schematically depicted in Fig. 5, various functions. (A) Mac-1 mediates leukocyte adhesion to the vascular wall by binding to ICAM-1 on endothelial cells, which, for example, is a precondition for chemotaxis-induced leukocyte extravasation and furthermore induces co-stimulatory effects [18,19]. (B) The binding of iC3b-opsonized bacteria or immune complexes induces phagocytosis and digestion and can induce respiratory bursts [20,21]. (C) The bivalent fibrinogen molecule is able to bind to two distinct cells and thereby mediates cross-bridging between cells. Fibrinogen binding to Mac-1 [22,23] can result in formation of leukocyte/leukocyte or leukocyte/platelet aggregates, which is described as a mechanism of neutrophil activation in unstable angina [24]. Furthermore, immobilized fibrinogen is abundant on injured vessel walls [25,26]. (D) Moreover, activated Mac-1 is able to bind factor X, whereby the conversion to factor Xa is accelerated. This represents an alternative, leukocyte-mediated pathway of initiation of the coagulation cascade.

What evidence is available that monocytes and in particular Mac-1, play a role in the clinical settings for which administration of abciximab demonstrated benefits? Acute coronary syndromes are associated with activation of monocytes and increased expression of adhesion molecules [27,28]. Coronary angioplasty leads to phagocyte-activation and Mac-1 upregulation and the extent of this upregulation affects the rate of restenosis [29–31]. Mac-1-deficient mice demonstrate a reduction of neointima formation after vessel injury [32]. Mac-1 upregulation is associated with an increase in procoagulant activity [33,34]. Furthermore, Mac-1 blockade may minimize reperfusion injury. Cats treated with a Mac-1-blocking mAb demonstrated a significant reduction of plasma creatine phosphokinase activity and endothelial dysfunction after experimental ischemia and reperfusion [35]. Reduction of postischemic cell damage was not only found in the heart but also in cerebral injury [36]. In addition, Seshiah et al. [37] provided evidence that monocytes trigger vascular smooth muscle cell apoptosis and thus may influence plaque rupture.

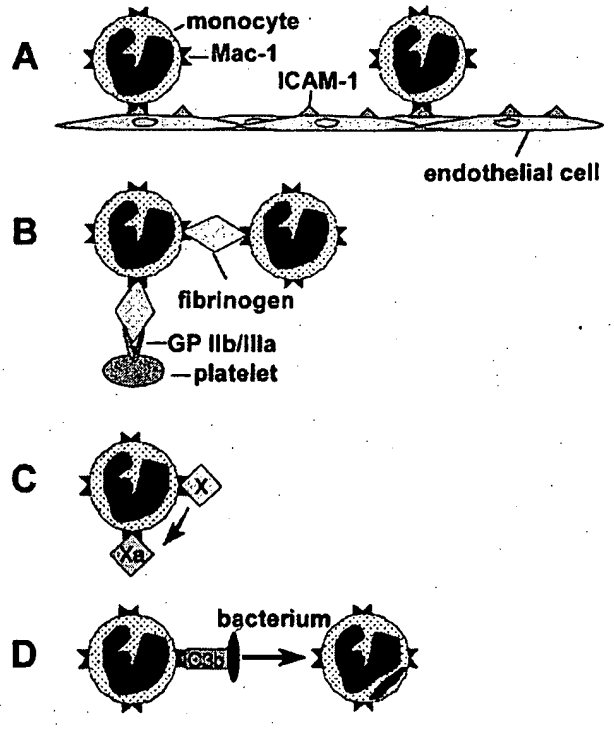

Fig. 5. Schematic drawing of Mac-1 functions that are inhibited by abciximab.

It has been demonstrated that abciximab binds to a continuous peptide sequence of the I-domain (also called A-domain) of the Mac-1 α-subunit [9]. In contrast, human murine domain-swapping experiments indicate that the binding site on GP IIb/IIIa is a discontinuous sequence of the β-subunit [38]. These findings reflect the different characters of these two receptors: While Mac-1 is a so-called I-domain integrin with a clearly defined ligand binding site in the α-subunit, GP IIb/IIIa is a member of the non-I-domain integrins. The precise tertiary structure of the latter has not been completely resolved yet and its complex- and conformation-dependent ligand binding pocket is probably assembled by sequences of both subunits [39]. Interestingly, a homologous metal ion-dependent adhesion site (MIDAS)-like motif containing the *DXSXS* motif is present in all β-subunits as well as in the I-domain binding sequence for abciximab [9]. Since the I-domain has also been proposed as the binding site for various adhesive ligands of Mac-1, [16,17] we hypothesized that abciximab binding to Mac-1 might inhibit the binding of several ligands.

The finding that abciximab inhibits the Mac-1-mediated factor X conversion provides an additional explanation for the overall clinical benefits of abciximab. Together with the recently described inhibition of platelet-mediated thrombin formation by abciximab, [40] this effect directly inhibits initiation of the coagulation cascade in addition to the anti-platelet and anti-leukocyte effects. Beyond the well-established blockade of GP IIb/IIIa, we describe anti-leukocyte and anticoagulative effects that may contribute to the overall clinical benefits of abciximab.

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References

[1] Frishman WH, Burns B, Atac B, Alturk N, Altajar B, Lerrick K. Novel antiplatelet therapies for treatment of patients with ischemic heart disease: inhibitors of the platelet glycoprotein IIb/IIIa integrin receptor. Am Heart J 1995;130:877–92.

[2] Lincoff AM, Califf RM, Topol EJ. Platelet glycoprotein IIb/IIIa receptor blockade in coronary artery disease. J Am Coll Cardiol 2000;35:1103–15.

[3] Kereiakes DJ, Runyon JP, Broderick TM, Shimshak TM. IIb's are not IIb's. Am J Cardiol 2000;85:23C–31C.

[4] Scarborough RM, Kleiman NS, Phillips DR. Platelet glycoprotein IIb/IIIa antagonists. What are the relevant issues concerning their pharmacology and clinical use? Circulation 1999;100:437–44.

[5] Topol EJ, Ferguson JJ, Weisman HF, Tcheng JE, Ellis SG, Kleiman NS, et al. Long-term protection from myocardial ischemic events in a randomized trial of brief integrin beta3 blockade with percutaneous coronary intervention. EPIC Investigator Group. Evaluation of platelet IIb/IIIa inhibition for prevention of ischemic complication. JAMA 1997;278:479–84.

[6] Topol EJ, Byzova TV, Plow EF. Platelet GPIIb-IIIa blockers. Lancet 1999;353:227–31.

[7] Tam SH, Sassoli PM, Jordan RE, Nakada MT. Abciximab (ReoPro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and alpha(v)beta3 integrins. Circulation 1998;98:1085–91.

[8] Kupatt C, Habazettl H, Hanusch P, Wichels R, Hahnel D, Becker BF, et al. c7E3Fab reduces postischemic leukocyte–thrombocyte interaction mediated by fibrinogen. Implications for myocardial reperfusion injury. Arterioscler Thromb Vasc Biol 2000;20:2226–32.

[9] Plescia J, Conte MS, VanMeter G, Ambrosini G, Altieri DC. Molecular identification of the cross-reacting epitope on alphaM beta2 integrin I domain recognized by anti-alphaIIb beta3 monoclonal antibody 7E3 and its involvement in leukocyte adherence. J Biol Chem 1998;273:20372–7.

[10] Thompson RD, Wakelin MW, Larbi KY, Dewar A, Asimakopoulos G, Horton MA, et al. Divergent effects of platelet–endothelial cell adhesion molecule-1 and beta 3 integrin blockade on leukocyte transmigration in vivo. J Immunol 2000;165:426–34.

[11] Coller BS. Potential non-glycoprotein IIb/IIIa effects of abciximab. Am Heart J 1999;138:S1–5.

[12] Coller BS. Binding of abciximab to alpha V beta 3 and activated alpha M beta 2 receptors: with a review of platelet–leukocyte interactions. Thromb Haemost 1999;82:326–36.

[13] Altieri DC, Edgington TS. A monoclonal antibody reacting with distinct adhesion molecules defines a transition in the functional state of the receptor CD11b/CD18 (Mac-1). J Immunol 1988;141: 2656–60.

[14] Simon DI, Xu H, Ortlepp S, Rogers C, Rao NK. 7E3 monoclonal antibody directed against the platelet glycoprotein IIb/IIIa cross-reacts with the leukocyte integrin Mac-1 and blocks adhesion to fibrinogen and ICAM-1. Arterioscler Thromb Vasc Biol 1997;17:528–35.

[15] Zhou L, Lee DH, Plescia J, Lau CY, Altieri DC. Differential ligand binding specificities of recombinant CD11b/CD18 integrin I-domain. J Biol Chem 1994;269:17075–9.

[16] Zhang L, Plow EF. Overlapping, but not identical, sites are involved in the recognition of C3bi, neutrophil inhibitory factor, and adhesive ligands by the alphaMbeta2 integrin. J Biol Chem 1996; 271:18211–6.

[17] Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. The leukocyte integrins. J Biol Chem 2000;275:23409–12.

[18] Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301–14.

[19] Schnitzler N, Haase G, Podbielski A, Lutticken R, Schweizer KG. A co-stimulatory signal through ICAM-beta2 integrin-binding potentiates neutrophil phagocytosis. Nat Med 1999;5:231–5.

[20] Xia Y, Vetvicka V, Yan J, Hanikyrova M, Mayadas T, Ross GD. The beta-glucan-binding lectin site of mouse CR3 (CD11b/CD18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to iC3b-opsonized target cells. J Immunol 1999;162:2281–90.

[21] Foreman KE, Bjornson AB. The alternative complement pathway promotes IgM antibody-dependent and -independent adherence of *Bacteroides* to polymorphonuclear leukocytes through CR3 and CR1. J Leukoc Biol 1994;55:603–11.

[22] Altieri DC, Bader R, Mannucci PM, Edgington TS. Oligospecificity of the cellular adhesion receptor Mac-1 encompasses an inducible recognition specificity for fibrinogen. J Cell Biol 1988;107: 1893–900.

[23] Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. Proc Natl Acad Sci U S A 1988;85:7734–8.

[24] Kuijper PH, Gallardo Torres HI, van der Linden JA, Lammers JW, Sixma JJ, Koenderman L, et al. Platelet-dependent primary hemostasis promotes selectin- and integrin-mediated neutrophil adhesion to damaged endothelium under flow conditions. Blood 1996;87: 3271–81.

[25] Altieri DC. Regulation of leukocyte–endothelium interaction by fibrinogen. Thromb Haemost 1999;82:781–6.

[26] Languino LR, Plescia J, Duperray A, Brian AA, Plow EF, Geltosky JE, et al. Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. Cell 1993;73: 1423–34.

[27] Jude B, Agraou B, McFadden EP, Susen S, Bauters C, Lepelley P, et al. Evidence for time-dependent activation of monocytes in the systemic circulation in unstable angina but not in acute myocardial infarction or in stable angina. Circulation 1994;90:1662–8.

[28] Mazzone A, De Servi S, Ricevuti G, Mazzucchelli I, Fossati G, Pasotti D, et al. Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. Circulation 1993;88:358–63.

[29] Mickelson JK, Lakkis NM, Villarreal-Levy G, Hughes BJ, Smith CW. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease? J Am Coll Cardiol 1996;28:345–53.

[30] Ikeda H, Nakayama H, Oda T, Kuwano K, Yamaga A, Ueno T, et al. Neutrophil activation after percutaneous transluminal coronary angioplasty. Am Heart J 1994;128:1091–8.

[31] De Servi S, Mazzone A, Ricevuti G, Fioravanti A, Bramucci E, Angoli L, et al. Granulocyte activation after coronary angioplasty in humans. Circulation 1990;82:140–6.

[32] Simon DI, Dhen Z, Seifert P, Edelman ER, Ballantyne CM, Rogers C. Decreased neointimal formation in Mac-1(−/−) mice reveals a role for inflammation in vascular repair after angioplasty. J Clin Invest 2000;105:293–300.

[33] Ott I, Neumann FJ, Kenngott S, Gawaz M, Schomig A. Procoagulant inflammatory responses of monocytes after direct balloon angioplasty in acute myocardial infarction. Am J Cardiol 1998;82:938–42.

[34] Ott I, Andrassy M, Zieglgansberger D, Geith S, Schomig A, Neumann FJ. Regulation of monocyte procoagulant activity in acute myocardial infarction: role of tissue factor and tissue factor pathway inhibitor-1. Blood 2001;97:3721–6.

[35] Ma XL, Tsao PS, Lefer AM. Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. J Clin Invest 1991;88:1237–43.

[36] Zhang ZG, Chopp M, Tang WX, Jiang N, Zhang RL. Postischemic treatment (2–4 h) with anti-CD11b and anti-CD18 monoclonal antibodies are neuroprotective after transient (2 h) focal cerebral ischemia in the rat. Brain Res 1995;698:79–85.

[37] Seshiah PN, Kereiakes DJ, Vasudevan SS, Lopes N, Su BY, Flavahan NA, et al. Activated monocytes induce smooth muscle cell death: role

of macrophage colony-stimulating factor and cell contact. Circulation 2002;105:174–80.
[38] Puzon-McLaughlin W, Kamata T, Takada Y. Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding pocket in integrin alpha(IIb)beta(3). J Biol Chem 2000;275:7795–802.
[39] Humphries MJ. Integrin structure. Biochem Soc Trans 2000;28: 311–39.
[40] Reverter JC, Beguin S, Kessels H, Kumar R, Hemker HC, Coller BS. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and "clinical restenosis". J Clin Invest 1996;98:863–74.

Inflammation and Restenosis in the Stent Era

Frederick G.P. Welt, Campbell Rogers

Abstract—The pathophysiology of restenosis involves early elements of direct injury to smooth muscle cells, deendothelialization, and thrombus deposition. Over time, this leads to smooth muscle cell proliferation/migration and extracellular matrix deposition. There is an increasing body of evidence to suggest that inflammation plays a pivotal role linking early vascular injury to the eventual consequence of neointimal growth and lumen compromise. The widespread use of coronary stents has fundamentally altered the vascular response to injury by causing a more intense and prolonged inflammatory state. Many of the cellular and molecular elements responsible for leukocyte recruitment have been elucidated, providing potential therapeutic targets for restenosis. This review seeks to provide an integrated view of the pathophysiology of restenosis that explains the central role of inflammation. **(*Arterioscler Thromb Vasc Biol.* 2002;22: 1769-1776.)**

Key Words: stenting ■ balloon angioplasty ■ restenosis ■ inflammation

Since the first reports of successful angioplasty of human coronary atherosclerotic lesions, restenosis has been encountered as a significant limitation to the long-term efficacy of the procedure. In their 1979 landmark publication "Non-Operative Dilatation of Coronary-Artery Stenosis," Gruntzig et al[1] reported that 6 of 32 patients undergoing successful initial angioplasty suffered restenosis, a rate of 19%. Subsequent large-scale registries documented a restenosis rate closer to 33%.[2] Early histological studies of specimens retrieved from human necropsy specimens recognized a predominantly fibrocellular response at sites of prior angioplasty.[3] Early animal studies revealed initial endothelial denudation, medial dissection, and platelet deposition as an immediate response to balloon injury and described late restenosis as a consequence of smooth muscle cell (SMC) proliferation and organized intraluminal thrombosis.[4,5] Based on these understandings, early attempts to limit restenosis focused on antithrombotic and antiproliferative agents, but there was limited success in animal models and no success in human trials.[6] In 1991, Forrester et al[7] proposed a paradigm for restenosis based on the vascular biology of wound healing and suggested 3 phases in the process: an inflammatory phase, a granulation or cellular proliferation phase, and a phase of remodeling involving extracellular matrix protein synthesis. Subsequent studies have supported a critical role for inflammatory cells in the restenotic process and have uncovered a critical role for the thrombotic cascade in the initial recruitment of these inflammatory cells. As this fundamental understanding of restenosis after balloon angioplasty has developed, however, the clinical practice of interventional cardiology has undergone a profound change with the advent of intracoronary stenting. What has been perhaps underappreciated in the evolving understanding of restenosis from animal model and clinical perspectives is the profound impact that a chronic indwelling stent has on the vascular biological response to coronary intervention, particularly the impact on the inflammatory response.

Studies of restenosis in humans are limited by the fact that direct tissue examination is only rarely possible. Therefore, indirect measures of site-specific pathophysiological processes are the mainstay of this research. Animal studies allow direct examination of tissue and the ability to vary experimental conditions. However, animal models are not perfect mirrors of human pathology, and as proof, there are numerous examples of therapies that are effective in animals but not in humans. Therefore, animal studies are best used to answer specific biological questions that give insight into human disease rather than to provide exact surrogates of human pathology. The present review seeks to provide an integrated view of the pathophysiology of restenosis explaining the central role of inflammation. Evidence from animal and human studies of the importance of inflammation is described; also described are mechanisms of inflammatory cell recruitment and how these processes might be targeted to prevent restenosis. In addition, we discuss how endovascular stenting fundamentally affects the pathophysiological responses to vascular injury, with emphasis on the inflammatory responses.

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Mechanisms of Leukocyte Recruitment and Infiltration

Leukocyte recruitment and infiltration occur at sites of vascular injury where the lining endothelial cells have been denuded and platelets and fibrin have been deposited. In vivo studies have shown that leukocytes and platelets colocalize at sites of hemorrhage, within atherosclerotic and postangioplasty restenotic lesions, and in areas of ischemia/reperfusion injury. This interaction between platelets and leukocytes appears to be critical to the inflammatory response.[8,9]

A paradigm of leukocyte attachment to and transmigration across surface-adherent platelets has been proposed.[10] The initial loose association to platelets and rolling of leukocytes is mediated by platelet P-selectin.[11] This is followed by their firm adhesion and transplatelet migration, processes that are dependent on the integrin class of adhesion molecules[10] The β_2 integrin molecule Mac-1 (CD11b/CD18) is present on neutrophils and monocytes and appears to be of central importance in leukocyte recruitment after vascular injury. In addition to promoting the accumulation of leukocytes at sites of vascular injury, the binding of platelets to neutrophils amplifies the inflammatory response by inducing neutrophil activation, upregulating cell adhesion molecule expression, and generating signals that promote integrin activation and chemokine synthesis. Interestingly, neutrophil-platelet and monocyte-platelet aggregates have been identified in the peripheral blood of patients with coronary artery disease and may be markers of disease activity.[12,13]

Also central to the recruitment of leukocytes to areas of vascular injury, chemokines are a group of chemoattractant cytokines produced by a variety of somatic cells, including SMCs, endothelial cells, and leukocytes. One such chemokine of the C-C class, monocyte chemoattractant protein (MCP)-1, participates in the recruitment of monocytes (as well as basophils and certain activated T cells).[14] The C-X-C chemokine, interleukin (IL)-8, plays a critical role in the recruitment of leukocytes to areas of vascular injury. It is well known that IL-8 is a pivotal cytokine in the recruitment of neutrophils.[15] More recent evidence suggests that the murine analogue of IL-8, KC, also plays a critical role in the recruitment of monocytes to injured areas.[16]

Human Evidence for Role of Inflammation in Restenosis

It has long been recognized that restenosis is not a case of accelerated atherosclerosis but is rather a distinct temporal and pathophysiological process. Yet evidence has emerged indicating that leukocytes play a central role in atherogenesis and restenosis. A role for leukocytes in the pathogenesis of atherosclerosis has long been accepted. This is largely based on numerous human histopathological studies in which leukocytes, mainly of the monocyte lineage, have been identified at all stages of development of the atherosclerotic plaque, from fatty streaks to mature atheroma.[17] Similar observations regarding restenosis have been hampered by the difficulty of obtaining human restenotic tissue. Farb et al[18] recently reported findings from pathological studies of 116 stents from 87 patients >90 days after the procedure. They found a strong link between the extent of medial damage, inflammation, and restenosis. Further crucial evidence linking leukocytes and restenosis has been provided by the study of Moreno et al,[19] in which the authors obtained tissue from directional atherectomy catheters at the time of angioplasty and found a strong positive correlation between the number of macrophages present in the tissue at the time of angioplasty and the subsequent propensity for restenosis.

Given the difficulty of obtaining human restenotic tissue, many investigators have looked at systemic markers of inflammation after angioplasty. Neumann et al[20] devised a technique in which they collected blood samples proximal to and just distal to the site of balloon dilatation in humans. They performed flow cytometry to determine the expression of the neutrophil adhesion molecules L-selectin and CD11b and found an upregulation of these markers of leukocyte activation after angioplasty, measured as a gradient between distal and proximal specimens. Mickelson et al[21] used systemic venous specimens from patients undergoing angioplasty and found flow cytometric evidence of the upregulation of CD11b on neutrophils and monocytes that correlated with a propensity for adverse clinical events. Inoue et al[22] extended these observations to demonstrate that elevated levels of neutrophil CD11b are predictive of future propensity for restenosis in patients undergoing balloon angioplasty. Pietersma et al[23] showed that IL-1 production by stimulated monocytes of blood from patients before angioplasty predicted later luminal loss, whereas activation of granulocytes, measured by CD66 levels, was inversely correlated with late loss. Cipollone et al[24] demonstrated upregulated levels of MCP-1 after percutaneous intervention in humans and found that MCP-1 levels were correlated with a risk for restenosis. More recently, Gaspardone et al[25] showed a correlation between C-reactive protein (a nonspecific inflammatory marker) after stent placement and a propensity for restenosis.

Animal Model Evidence for Role of Inflammation in Restenosis

In experimental animal models, cell adhesion molecules critical for leukocyte recruitment have been found to be upregulated by an atherogenic diet,[26–28] diabetes,[29] and increased shear stress.[30] In addition, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and major histocompatibility complex class II antigens have all been shown to be upregulated in a sustained fashion after balloon injury in the rabbit.[31] Our laboratory has demonstrated that in animal models in which an endovascular stent is placed to produce deep trauma to the vessel, a particularly brisk early inflammatory response is induced with abundant surface adherent leukocytes of monocyte and granulocyte lineage.[32,33] Days and weeks later, macrophages invade the forming neointima and are observed clustering around stent struts, forming giant cells. Blockade of early monocyte recruitment with anti-inflammatory agents results in reduced late neointimal thickening.[32,34,35] As a corollary, a linear relationship exists between tissue monocyte content and neointimal area, suggesting a pivotal causal role for monocytes in restenosis.[32] Activated macrophages are thought to influence vascular repair by producing a variety of mediators, including members of the interleukin family, tumor necrosis

factor, MCP-1, and growth factors, such as platelet-derived growth factors, basic fibroblast growth factor, and heparin-binding epidermal growth factor.[36]

Several studies have also shown infiltration of neutrophils within the arterial wall after vascular injury.[37–39] We recently reported the presence of neutrophils within the media and neointima of injured rabbit iliac arteries and demonstrated concomitant reductions in neutrophil number and medial smooth muscle proliferation with administration of anti-inflammatory agents.[33] The mechanisms by which neutrophils may affect vascular repair are not fully understood. Although neutrophils are not known to secrete growth factors per se, they do contribute to tissue injury through the release of reactive oxygen species and proteases.[36] Neutrophils are known to secrete cytokines, including IL-1, tumor necrosis factor-α, and IL-6.[40] In addition, it has been reported that rabbit vascular smooth cells are stimulated to proliferate when they are cocultured with neutrophils or neutrophil-conditioned media.[41]

Differences Between Balloon and Stent Injury

A major advance in the care of the patient undergoing cardiac intervention in the past decade has been the introduction of the coronary stent. This device has dramatically improved acute results and has reduced the incidence of subsequent restenosis. Still, in-stent restenosis, although less frequent, affects ≈20% of patients and is often recalcitrant and costly to treat. What has emerged from systematic investigation in human and animal studies are profound differences between vascular biological responses to balloon- and stent-induced injury.

Human Studies of the Pathophysiology of In-Stent Versus Balloon Angioplasty Restenosis

The use of intravascular ultrasound to study restenotic lesions has been invaluable in the elucidation of mechanisms of restenosis. Studying restenosis after balloon angioplasty, Mintz et al[42] used intravascular ultrasound to determine the contributions of neointimal hyperplasia and negative remodeling. They found that although negative remodeling (as measured by external elastic membrane area) and neointimal hyperplasia (as measured by plaque plus media cross-sectional area) contributed to restenosis, negative remodeling contributed substantially more (Figure 1). Angiographic analysis of the 2 first pivotal studies of coronary stents in humans (Stent Restenosis Study [STRESS][43] and Belgian Netherlands Stent Study [BENESTENT][44]) revealed distinct qualitative and quantitative differences between balloon-injured and stented arteries. Stented arteries experienced a much larger initial lumen gain, which was presumably due to the rigid scaffolding provided by the stent that prevents acute elastic recoil. At follow-up, the luminal area was greater and binary restenosis was less in stented arteries than in balloon-angioplastied arteries. However, somewhat counterintuitively, late loss (lumen immediately after follow-up minus lumen at follow-up) was greater in stented arteries. Tying these observations together, stents incur greater neointimal growth, with their net benefit being attributable to their larger initial lumen gain and prevention of remodeling. This was confirmed with a serial intravascular ultrasound study conducted by Hoffman et al[45] (Figure 2).

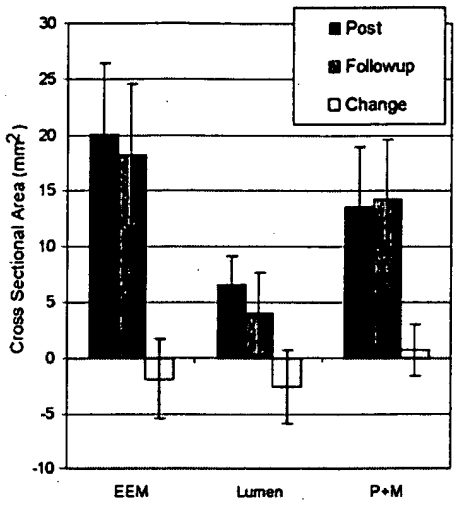

Figure 1. Graph of intravascular ultrasound measurements after balloon angioplasty. The arterial size, as measured by the cross-sectional area of the external elastic membrane (EEM), shrinks (remodels) over time, contributing more to the decrement in lumen size than does neointimal growth, as measured by the cross-sectional area of the plaque plus media (P+M). Adapted from Mintz et al.[42]

Although direct measures of inflammation at the site of clinical percutaneous intervention are virtually impossible, evidence has been gathered to suggest that there are differences in the inflammatory responses to stent placement and balloon angioplasty. Inoue et al[46] have used flow cytometry to measure CD11b expression on neutrophils after percutaneous coronary intervention and have found substantially higher levels on neutrophils from patients undergoing stent implantation compared with patients

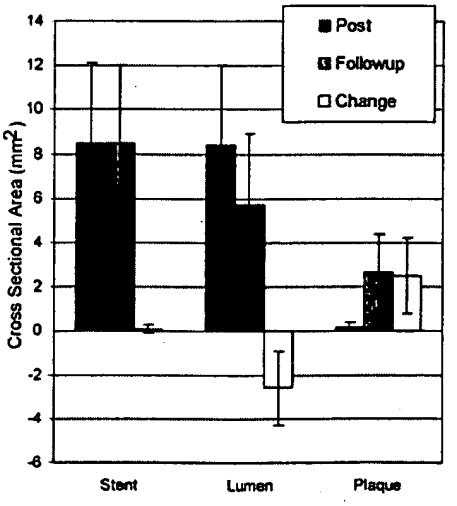

Figure 2. Graph of intravascular ultrasound measurements after stent placement. The arterial size, as measured by stent cross-sectional area, stays fixed over time, indicating no remodeling. The decrement in lumen size is entirely caused by neointimal growth, as measured by plaque cross-sectional area. Adapted from Hoffmann et al.[45]

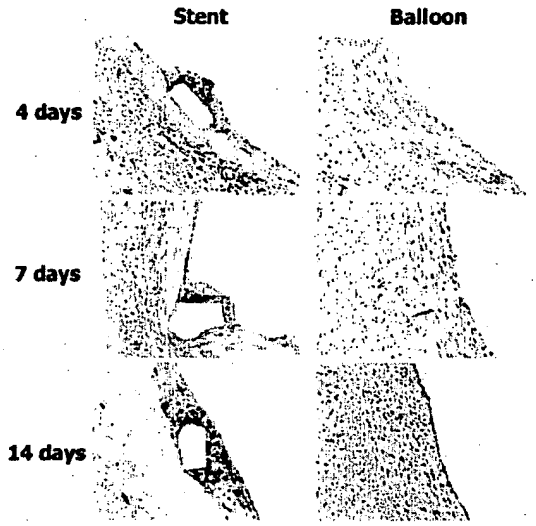

Figure 3. Immunohistochemical identification of macrophages within primate iliac arteries injured with either stents or balloon injury alone. Note that there is a steady accumulation of macrophages within stented segments with clustering of macrophages surrounding stent struts, whereas there is virtually no accumulation within the balloon-injured segments. From Horvath et al.[52]

undergoing balloon angioplasty alone. This increased inflammatory response may help to explain the larger neointimal growth seen in stented arteries.

Animal Studies of the Pathophysiology of In-Stent Versus Balloon Angioplasty Restenosis

Several animal studies have demonstrated marked differences in response to vascular injury between balloon- and stent-induced injury, with specific emphasis on differences in the inflammatory response. Heparin, an archetypal modulator of vascular repair in animal models, has long been known to reduce neointimal growth after vascular injury.[47,48] Heparin is equally effective at reducing neointimal hyperplasia after balloon injury or stent implantation.[49,50] However, our laboratory has shown that maximal inhibition of neointimal hyperplasia in stented arteries requires continuous heparin administration for the duration of the experiment, whereas transient early heparin therapy after balloon injury is just as effective as chronic administration.[50] An explanation of this difference is suggested by further studies from our laboratory. In stented arteries, there is abundant recruitment of macrophages within the neointima (Figure 3). Inhibition of macrophage accumulation parallels reduction in neointimal growth.[32] In contrast, in balloon-injured rabbit iliac arteries, a model devoid of macrophage infiltration (Figure 3), neutrophil infiltration has been documented within hours of injury. Inhibition of neutrophil infiltration is correlated with an inhibition of medial SMC proliferation.[33] To elucidate the mechanisms behind these differences, we determined mRNA levels of the monocyte chemokine MCP-1 and the neutrophil chemokine IL-8 at sites of vascular injury by using semiquantitative reverse transcriptase–polymerase chain reaction. In balloon injury, there was only transient expression (lasting only hours) of MCP-1 and IL-8. In contrast, in stented arteries, there was sustained expression of IL-8 and, more prominently, MCP-1 as late as 14 days.[51]

We have been able to exploit these differences in inflammatory response with leukocyte-specific therapies. In a primate iliac artery model, monocyte-specific blockade, achieved via blockade of the MCP-1 receptor CCR2, was effective at reducing neointimal hyperplasia within stented segments of the arteries.[52] In contrast, blockade of CCR2 was not effective against neointimal hyperplasia within balloon-injured segments. For balloon-injured segments, neutrophil blockade, achieved by targeting the leukocyte β_2-integrin β-subunit CD18, was required to reduce neointimal hyperplasia. These data suggest not only that leukocyte infiltration is causally related to neointimal hyperplasia after balloon- or stent-induced injury but also that temporal and spatial patterns of leukocyte infiltration vary with different forms of arterial injury.

An Integrated View of In-Stent Restenosis

Figure 4 illustrates an integrated view of the pathophysiological processes underlying in-stent restenosis. Figure 4A illustrates a mature atherosclerotic plaque. The initial events immediately after stent placement result in deendothelialization and the deposition of a layer of platelets and fibrin at the injured site (Figure 4B). Activated platelets express adhesion molecules such as P-selectin and glycoprotein (GP) Ibα, which attach to circulating leukocytes via platelet receptors such as P-selectin glycoprotein ligand (Figure 4C with inset) and begin a process of rolling along the injured surface. Under the influence of cytokines (Figure 4C), leukocytes bind tightly to the leukocyte integrin (ie, Mac-1) class of adhesion molecules via direct attachment to platelet receptors such as GP Ibα and through cross-linking with fibrinogen to the GP IIb/IIIa receptor (Figure 4D, inset). The migration of leukocytes across the platelet-fibrin layer and into the tissue is driven by chemical gradients of cytokines released from SMCs and resident leukocytes (Figure 4C and 4D). Growth factors are released from platelets, leukocytes, and SMCs, which influence the proliferation and migration of SMCs from the media into the neointima (Table, Figure 4D). The resultant neointima consists of SMCs, extracellular matrix, and macrophages recruited over several weeks (Figure 4E). Over even longer periods of time (Figure 4F), there is a shift to fewer cellular elements with, and greater production of, extracellular matrix. In addition, there is eventual reendothelialization of at least part of the injured vessel surface.

Implications for Antirestenotic Therapies

The data presented in the present review suggest that an anti-inflammatory approach may be an effective way to suppress neointimal growth and restenosis after percutaneous intervention in humans. Indeed, a shared feature of many promising therapies in clinical trials currently is an anti-inflammatory potential. For example, sirolimus, which has shown remarkable efficacy against restenosis in a coated stent design,[53] is a known inhibitor of regulatory elements of the cell cycle. However, it also possesses important anti-inflammatory properties, as evidenced by its initial develop-

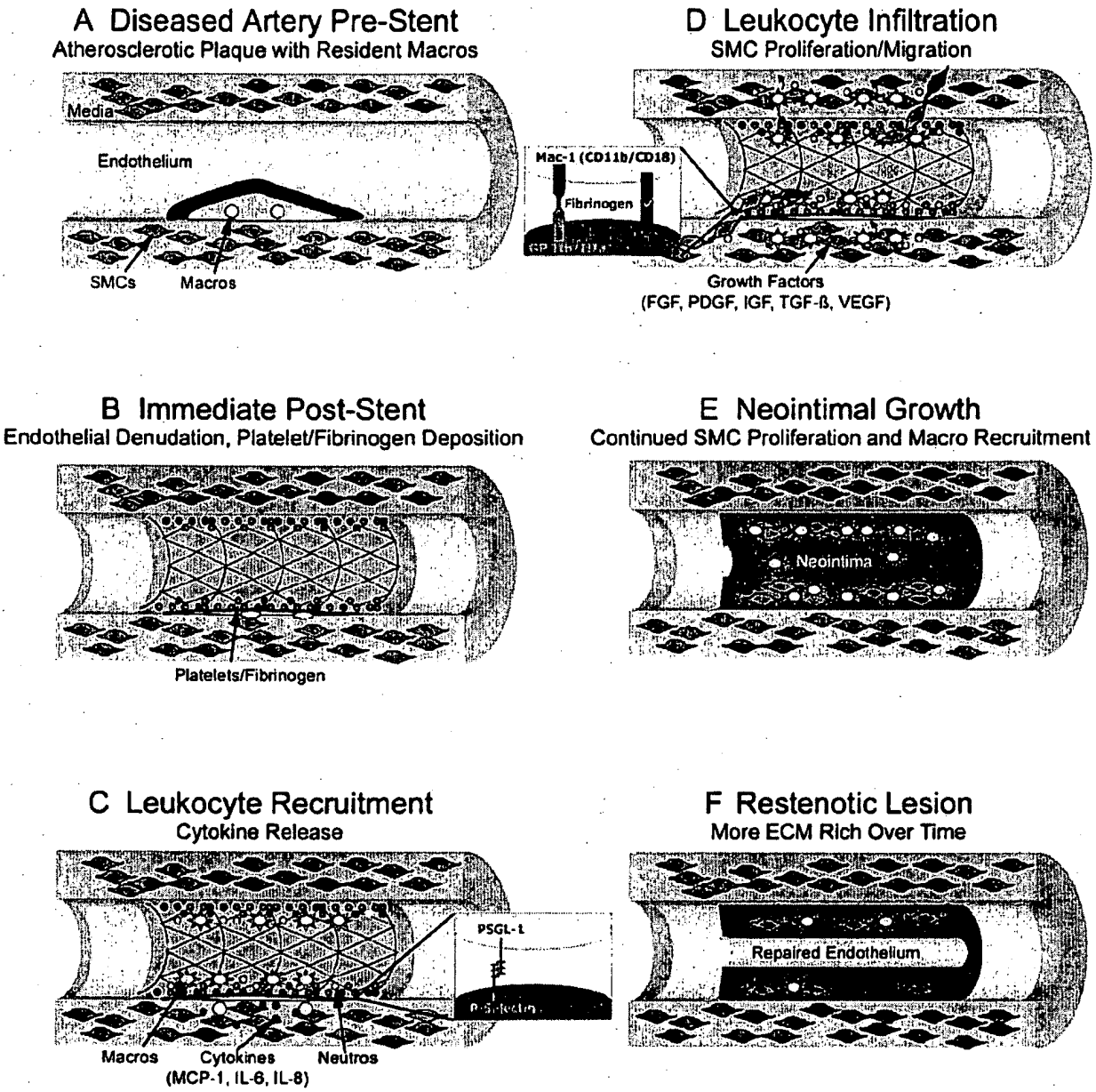

Figure 4. A, Mature atherosclerotic plaque before intervention. B, Immediate result of stent placement with endothelial denudation and platelet/fibrinogen deposition. C and D, Leukocyte recruitment, infiltration, and SMC proliferation and migration in the days after injury. E, Neointimal thickening in the weeks after injury, with continued SMC proliferation and monocyte recruitment. F, Long-term (weeks to months) change from a predominantly cellular to a less cellular and more ECM-rich plaque.

ment as an antifungal agent and its current use as an immunomodulatory agent in the treatment of renal transplant rejection.[54] In a porcine model of stent injury, sirolimus-coated stents, compared with bare metal stents, were associated with reduced vessel wall protein expression of the cytokines MCP-1 and IL-6.[55] Similarly, the microtubule stabilizer paclitaxel, a promising therapy for restenosis via stent-based delivery, is known to interfere with SMC proliferation and migration through its effect on microtubules.[54] However, there are also data to suggest that paclitaxel affects leukocyte function. Interestingly, this appears at least in part to be mediated through the interference of cytoskeletal interactions with the integrin class of adhesion molecules.[56] The effects of brachytherapy on inflammation have not been studied in detail, although one report suggests that delayed endothelialization after brachytherapy in a porcine model may actually increase the inflammatory burden.[57]

The data presented in the present review also suggest that there are important differences in the temporal and spatial pattern of inflammation between stent and balloon injury that must be taken into account when antirestenotic therapies are designed. Specifically, the inflammatory response engendered by a stent is prolonged and rich in cells of the monocyte/macrophage lineage. Therefore, anti-inflammatory therapies may have to be delivered over a prolonged period of time and also must include anti-macrophage activity. This requirement for prolonged activity makes all the more appealing the use of the stent itself as a platform for delivery.

Major Growth Factors/Cytokines Involved in the Restenotic Process

Growth Factor	Source (Within Vessel Wall)	Putative Action
PDGF A and B	Platelets	SMC migration and proliferation
	Endothelial cells	
	SMCs	
FGF (acidic and basic)	SMCs	SMC proliferation
Insulin-like growth factors	SMCs	SMC proliferation especially as comitogen with PDGF and FGF
Transforming growth factor β	Endothelial cells	SMC proliferation and inhibition
	SMCs	
Vascular endothelial growth factor	Endothelial cells	Endothelialization and angiogenesis
Cytokine	**Source (Within Vessel Wall)**	**Classification and Putative Action**
MCP-1	Macrophages	A C-C chemokine predominately involved in monocyte recruitment
	SMCs	
	Endothelial cells	
	Fibroblasts	
IL-8	Macrophages, T-cells, PMNs	A C-X-C chemokine predominately involved in neutrophil and monocyte recruitment
	SMCs	
	Endothelial cells	
	Fibroblasts	
IL-6	Macrophages, T-cells, PMNs	A member of the IL-6 family of cytokines involved in acute phase reactant expression; specific vascular effects unknown.
	SMCs	
	Endothelial cells	
	Fibroblasts	

PDGF indicates platelet-derived growth factor; FGF, fibroblast growth factor; PMN, polymorphonuclear leukocytes..

A final word should be said regarding the lack of efficacy of prior anti-inflammatory drugs in human trials of restenosis. In a broad sense, one can implicate either a lack of understanding of the biological responses to injury, use of the wrong drug, or an incomplete understanding of pharmacokinetics and pharmacodynamics. First, many of these trials were conducted in an era of predominant balloon angioplasty rather than stenting, which, as we have described in the present review, causes a fundamental change in the nature of the vascular biological response to injury. Heparin provides an example for which pharmacokinetics and pharmacodynamics may be the issue. Heparin is the archetypical modulator of vascular repair after vascular injury in a variety of animal models. It has long been known that heparin inhibits SMC proliferation and neointimal hyperplasia independent of its anticoagulant properties.[58] Multiple studies have also demonstrated that heparin also is a powerful anti-inflammatory agent and that this characteristic is intimately related to its antiproliferative properties in animal studies of vascular injury.[33,59] However, human trials of subcutaneous heparin after percutaneous intervention have proven ineffective in preventing restenosis.[60] An explanation of this paradox is suggested by data from prior animal studies suggesting that the efficacy of heparin against neointimal growth is critically dependent on the type of vascular injury imposed[61] and the duration and frequency of heparin administration.[50] These data suggest that human studies of heparin after percutaneous intervention may have suffered from an insufficient dosing interval and duration of administration. Although less completely studied than heparin, it is possible that pharmacokinetic and pharmacodynamic issues may also lie behind the failure of tranilast, a drug showing efficacy in animal models[62] but not in a large-scale human trial.[63]

Conclusions

An evolving understanding of the pathogenesis of restenosis has placed inflammation at the center of the process. Much of our early understanding of this process was based on studies of balloon angioplasty. It is imperative to understand that the placement of a stent, now the predominant therapy in humans, profoundly alters all elements of vascular repair, particularly inflammatory processes. These differences must be understood and incorporated into the development of antirestenotic therapies in the stent era.

References

1. Gruntzig AR, Senning A, Siegenthaler WE. Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. *N Engl J Med.* 1979;301:61–68.
2. Bourassa MG, Wilson JW, Detre KM, Kelsey SF, Robertson T, Passamani ER. Long-term follow-up of coronary angioplasty: the 1977–1981 National Heart, Lung, and Blood Institute registry. *Eur Heart J.* 1989;10(suppl G):36–41.
3. McBride W, Lange RA, Hillis LD. Restenosis after successful coronary angioplasty: pathophysiology and prevention. *N Engl J Med.* 1988;318:1734–1737.

4. Faxon DA, Weber VJ, Haudenschild C, Gottsman SB, McGovern WA, Ryan TJ. Acute effects of transmural angioplasty in three experimental models of atherosclerosis. *Arteriosclerosis.* 1982;2:125–133.
5. Faxon DP, Sanborn TA, Weber VJ, Haudenschild C, Gottsman SB, McGovern WA, Ryan TJ. Restenosis following transluminal angioplasty in experimental atherosclerosis. *Arteriosclerosis.* 1984;4:189–195.
6. Faxon DP, Sanborn TA, Haudenschild CC, Ryan TJ. Effect of antiplatelet therapy on restenosis after experimental angioplasty. *Am J Cardiol.* 1984; 53:72C–76C.
7. Forrester JS, Fishbein M, Helfant R, Fagin J. A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. *J Am Coll Cardiol.* 1991;17:758–769.
8. Marcus AJ. Thrombosis and inflammation as multicellular processes: significance of cell-cell interactions. *Semin Hematol.* 1994;31:261–269.
9. Libby P, Simon DI. Inflammation and thrombosis: the clot thickens. *Circulation.* 2001;103:1718–1720.
10. Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood.* 1996;88:146–157.
11. Yeo EL, Sheppard JA, Feuerstein IA. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood.* 1994;83:2498–2507.
12. Ott I, Neumann FJ, Gawaz M, Schmitt M, Schomig A. Increased neutrophil-platelet adhesion in patients with unstable angina. *Circulation.* 1996;94:1239–1246.
13. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB, Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol.* 1998;31:352–358.
14. Rollins BJ. Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. *Mol Med Today.* 1996;2: 198–204.
15. Webb LMC, Ehrengruber MU, Clark-Lewis I, Baggiolini M. Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc Natl Acad Sci U S A.* 1993;90:7158–7162.
16. Huo Y, Weber C, Forlow SB, Sperandio M, Thatte J, Mack M, Jung S, Littman DR, Ley K. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J Clin Invest.* 2001;108:1307–1314.
17. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
18. Farb A, Weber DK, Kolodgie FD, Burke AP, Virmani R. Morphological predictors of restenosis after coronary stenting in humans. *Circulation.* 2002;105:2974–2980.
19. Moreno PR, Bernardi VH, Lopez-Cuellar J, Newell JB, McMellon C, Gold HK, Palacios IF, Fuster V, Fallon JT. Macrophage infiltration predicts restenosis after coronary intervention in patients with unstable angina. *Circulation.* 1996;94:3098–3102.
20. Neumann F-J, Ott I, Gawaz M, Puchner G, Schomig A. Neutrophil and platelet activation at balloon-injured coronary artery plaque in patients undergoing angioplasty. *J Am Coll Cardiol.* 1996;27:819–824.
21. Mickelson JK, Lakkis NM, Villarreal-Levy G, Hughes B, Smith CW. Leukocyte activation with platelet adhesion after coronary angioplasty: a mechanism for recurrent disease. *J Am Coll Cardiol.* 1996;28:345–353.
22. Inoue T, Sakai Y, Morooka S, Hayashi T, Takayanagi K, Takabatake Y. Expression of polymorphonuclear leukocyte adhesion molecules and its clinical significance in patients treated with percutaneous transluminal coronary angioplasty. *J Am Coll Cardiol.* 1996;28:1127–1133.
23. Pietersma A, Kofflard M, de Wit LEA, Stinjen T, Koster JF, Serruys P, Sluiter W. Late lumen loss after coronary angioplasty is associated with the activation status of circulating phagocytes before treatment. *Circulation.* 1995;91:1320–1325.
24. Cipollone F, Marini M, Fazia M, Pini B, Iezzi A, Reale M, Paloscia L, Materazzo G, D'Annunzio E, Conti P, Chiarelli F, Cuccurullo F, Mezzetti A. Elevated circulating levels of monocyte chemoattractant protein-1 in patients with restenosis after coronary angioplasty. *Arterioscler Thromb Vasc Biol.* 2001;21:327–334.
25. Gaspardone A, Crea F, Versaci F, Tomai F, Pellegrino A, Chiariello L, Gioffre PA. Predictive value of C-reactive protein after successful coronary-artery stenting in patients with stable angina. *Am J Cardiol.* 1998; 82:515–518.
26. Cybulsky MI, Gimbrone MAJ. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science.* 1991;251: 788–791.
27. Li H, Cybulsky M, Gimbrone MA, Libby P. An atherogenic diet induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit endothelium. *Arterioscler Thromb.* 1992;13:197–204.
28. Li H, Cybulsky MI, Gimbrone MA Jr, Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. *Am J Pathol.* 1993;143:1551–1559.
29. Richardson M, Hadcock SJ, DeReske M, Cybulsky MI. Increased expression in vivo of VCAM-1 and E-selectin by the aortic endothelium of normolipemic and hyperlipemic diabetic rabbits. *Arterioscler Thromb.* 1994;14:760–769.
30. Walpola PL, Gotlieb AI, Cybulsky MI, Langille L. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. *Arterioscler Thromb Vasc Biol.* 1995;15:2–10.
31. Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI, Libby P. Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. *Circulation.* 1993;88:1788–1803.
32. Rogers C, Welt FGP, Karnovsky MJ, Edelman ER. Monocyte recruitment and neointimal hyperplasia in rabbits: coupled inhibitory effects of heparin. *Arterioscler Thromb Vasc Biol.* 1996;16:1312–1318.
33. Welt FGP, Edelman ER, Simon DI, Rogers C. Neutrophil, not macrophage, infiltration precedes neointimal thickening in balloon-injured arteries. *Arterioscler Thromb Vasc Biol.* 2000;20:2553–2558.
34. Rogers C, Edelman ER, Simon DI. A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proc Natl Acad Sci U S A.* 1998;95: 10134–10139.
35. Mori E, Komori K, Yamaoka T, Tanii M, Kataoka C, Takeshita A, Usui M, Egashira K, Sugimachi K. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. *Circulation.* 2002;105:2905–2910.
36. Libby P, Schwartz D, Brogi E, Tanaka H, Clinton S. A cascade model for restenosis. *Circulation.* 1992;86(suppl III):III-47–III-52.
37. Jorgensen L, Grothe AG, Groves HM, Kinlough-Rathbone RL, Richardson M, Mustard JF. Sequence of cellular responses in rabbit aortas following one and two injuries with a balloon catheter. *Br J Exp Pathol.* 1988;69:473–486.
38. Richardson M, Hatton MW, Buchanan MR, Moore S. Wound healing in the media of the normolipemic rabbit carotid artery injured by air drying or by balloon catheter de-endothelialization. *Am J Pathol.* 1990;137: 1453–1465.
39. Kockx MM, De Meyer GR, Jacob WA, Bult H, Herman AG. Triphasic sequence of neointimal formation in the cuffed carotid artery of the rabbit. *Arterioscler Thromb.* 1992;12:1447–1457.
40. Lloyd AR, Oppenheim JJ. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol Today.* 1992;13:169–172.
41. Cole CW, Makhoul RG, McCann RL, O'Malley MK, Hagen PO. A neutrophil derived factor(s) stimulates [3H]thymidine incorporation by vascular smooth muscle cells in vitro. *Clin Invest Med.* 1988;11:62–67.
42. Mintz GS, Popma JJ, Pichard AD, Kent KM, Satler LF, Wong C, Hong MK, Kovach JA, Leon MB. Arterial remodeling after coronary angioplasty: a serial intravascular ultrasound study. *Circulation.* 1996;94: 35–43.
43. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn IM, Detre K, Veltri L, Ricci DR, Nobuyoshi M, Cleman MW, Heuser RR, Almond D, Teirstein PS, Fish RD, Colombo A, Brinker J, Moses J, Shaknovich A, Hirshfeld J, Bailey S, Ellis S, Rake R, Goldberg S. A randomized comparison of coronary artery-stent placement and balloon angioplasty in the treatment of coronary artery disease. *N Engl J Med.* 1994;331:496–501.
44. Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, Emanuelsson H, Marco J, Legrand V, Materne P, Belardi J, Sijwart U, Colombo A, Goy J, van den Heuvel P, Delcan J, Morel M. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. *N Engl J Med.* 1994;331:489–495.
45. Hoffmann R, Mintz GS, Dussaillant GR, Popma JJ, Pichard AD, Satler LF, Kent KM, Griffin J, Leon MB. Patterns and mechanisms of in-stent restenosis: a serial intravascular ultrasound study. *Circulation.* 1996;94: 1247–1254.
46. Inoue T, Sohma R, Miyazaki T, Iwasaki Y, Yaguchi I, Morooka S. Comparison of activation process of platelets and neutrophils after cor-

onary stent implantation versus balloon angioplasty for stable angina pectoris. *Am J Cardiol.* 2000;86:1057–1062.
47. Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury: II. Inhibition of smooth muscle cell growth by heparin. *Lab Invest.* 1985;52:611–616.
48. Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury, IV: heparin inhibits rat smooth muscle cell mitogenesis and migration. *Circ Res.* 1986;58:839–845.
49. Rogers C, Edelman ER. Controlled release of heparin reduces neointimal hyperplasia in stented rabbit arteries: ramifications for local therapy. *J Interv Card.* 1992;5:195–202.
50. Edelman ER, Karnovsky MJ. Contrasting effects of the intermittent and continuous administration of heparin in experimental restenosis. *Circulation.* 1994;89:770–776.
51. Paolini JF, Kjelsberg MA, Edelman ER, Rogers CDK. Sustained expression of chemokines monocyte chemoattractant protein-1 and interleukin-8 after stent- but not balloon-induced arterial injury. *J Am Coll Cardiol.* 2000;35:15. Abstract.
52. Horvath C, Welt FG, Nedelman M, Rao P, Rogers C. Targeting CCR2 or CD18 inhibits experimental in-stent restenosis in primates: inhibitory potential depends on type of injury and leukocytes targeted. *Circ Res.* 2002;90:488–494.
53. Morice MC, Serruys PW, Sousa JE, Fajadet J, Ban Hayashi E, Perin M, Colombo A, Schuler G, Barragan P, Guagliumi G, Molnar F, Falotico R. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med.* 2002;346:1773–1780.
54. Oberhoff M, Herdeg C, Baumbach A, Karsch KR. Stent-based antirestenotic coatings (sirolimus/paclitaxel). *Catheter Cardiovasc Interv.* 2002; 55:404–408.
55. Suzuki TM, Kopia GP, Hayashi S, Bailey LRA, Llanos GP, Wilensky RM, Klugherz BDM, Papandreou GP, Narayan PP, Leon MBM, Yeung ACM, Tio FM, Tsao PSP, Falotico RP, Carter AJD. Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. *Circulation.* 2001;104:1188–1193.
56. Zhou X, Li J, Kucik DF. The microtubule cytoskeleton participates in control of beta2 integrin avidity. *J Biol Chem.* 2001;276:44762–44769.
57. Kollum M, Cottin Y, Chan RC, Kim H, Bhargava B, Vodovotz Y, Waksman R. Delayed re-endothelialization and T-cell infiltration following intracoronary radiation therapy in the porcine model. *Int J Radiat Oncol Biol Phys.* 2001;50:495–501.
58. Guyton JR, Rosenberg RD, Clowes AW, Karnovsky MJ. Inhibition of rat arterial smooth muscle cell proliferation by heparin: in vivo studies with anticoagulant and nonanticoagulant heparin. *Circ Res.* 1980;46:625–634.
59. Rogers C, Karnovsky MJ, Edelman ER. Heparin's inhibition of monocyte adhesion to experimentally injured arteries matches its antiproliferative effects. *Circulation.* 1993;88(suppl I):I-370. Abstract.
60. Ellis SG, Roubin GS, Wilentz J, Douglas JS Jr, King SB III. Effect of 18- to 24-hour heparin administration for prevention of restenosis after uncomplicated coronary angioplasty. *Am Heart J.* 1989;117:777–782.
61. Rogers C, Karnovsky MJ, Edelman ER. Inhibition of experimental neointimal hyperplasia and thrombosis depends on the type of vascular injury and the site of drug administration. *Circulation.* 1993;88:1215–1221.
62. Ishiwata S, Verheye S, Robinson KA, Salame MY, de Leon H, King SB III, Chronos NA. Inhibition of neointima formation by tranilast in pig coronary arteries after balloon angioplasty and stent implantation. *J Am Coll Cardiol.* 2000;35:1331–1337.
63. SoRelle R. Late-breaking clinical trials at the American Heart Association's Scientific Sessions 2001. *Circulation.* 2001;104:E9046–E9048.

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THROMBOSIS RESEARCH

ORIGINAL ARTICLE

Mechanisms Involved in the Inhibition of Neointimal Hyperplasia by Abciximab in a Rat Model of Balloon Angioplasty

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Abstract

Monoclonal antibodies raised against β_3 integrin are able to inhibit the binding of ligands to certain β_3 integrins such as $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa complex) and $\alpha_v\beta_3$ (vitronectin receptor) and as such are inhibitors of platelet aggregation and smooth muscle cell (SMC) migration, both of which are involved in neointimal hyperplasia. The present study was designed to explore the detailed mechanisms of abciximab (Reopro), a monoclonal antibody (mAb) raised against $\alpha_{IIb}\beta_3$ integrin in neointimal hyperplasia. In this study, carotid arteries of Wistar rats were damaged, and neointimal hyperplasia and lumen occlusion was determined at different time points. Abciximab was administered intravenously by an implanted osmotic pump. Abciximab (0.25 mg/kg/day) time-dependently inhibited both neointimal hyperplasia and lumen occlusion after angioplasty in carotid arteries of rats. Furthermore, the electromicrographs highlighted that SMCs were phenotypically different from the typical contractile, spindle-shaped SMCs normally seen in uninjured vessel walls. Platelet-derived growth factor (PDGF)-BB was strongly produced in thrombus formation and neointimal SMCs after angioplasty, while abciximab significantly reduced PDGF-BB expression in vessel lumens and neointimal SMCs after angioplasty. Balloon angioplasty caused a significant increase of nitrate and cyclic GMP as compared with sham-operated rats. Infusion of abciximab (0.25 mg/kg/day) did not significantly change. Furthermore, the plasma level of thromboxane B_2 (TxB_2) obviously increased after angioplasty, while abciximab markedly suppressed the elevation of plasma TxB_2 concentration. The results indicate that abciximab effectively prevents neointimal hyperplasia, possibly through the following 2 mechanisms: (1) Abciximab binds to $\alpha_{IIb}\beta_3$ integrin on platelet membranes resulting in inhibition of platelet adhesion, secretion, and aggregation in injured arteries, followed by inhibition of thromboxane A_2 formation and PDGF-BB release from platelets. (2) Abciximab may also bind to $\alpha_v\beta_3$ integrin on SMCs, thus, subsequently inhibiting cell migration and proliferation.

Key Words: Abciximab; β_3 integrin; Neointimal hyperplasia; Balloon angioplasty; PDGF-BB

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Vessel wall injury produced by passage of a balloon catheter through the lumen of an artery initiates a sequence of vascular

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smooth muscle cell (SMCs) responses including stimulation of SMC migration and proliferation, which leads to intimal hyperplasia [1]. This process contributes to the pathogenesis of several cardiovascular disorders, including atherosclerosis, and restenosis after angioplasty [1]. Although the cellular mechanisms of neointimal hyperplasia are not fully understood, the process appears to involve early platelet accumulation, replication of medial vascular SMCs, migration into the intima of replicating and into the media of nonreplicating vascular SMCs, and chronic replication of intimal vascular SMCs in response to mitogens and growth factors [2]. A number of growth factors, including fibroblast growth factor and platelet-derived growth factor (PDGF), are released at the site of vascular injury, which indicates the significance of these mitogenic and chemotactic stimuli in vascular proliferation [3].

Arterial injury exposes the subendothelial matrix including fibronectin and collagen which, in turn, prompts platelet adhesion and accumulation [4]. Therefore, platelet degranulation products may play an important role in initiation of neointimal hyperplasia. Some evidences have been provided that platelets are involved in these processes. For example, thrombocytopenic animals exhibited significantly less intimal thickening after balloon angioplasty than did normal controls [5]. However, clinical trials using various classic antiplatelet drugs presently available showed no beneficial effect [6].

More potent antiplatelet agents such as inhibitors of the platelet fibrinogen receptors (glycoprotein IIb/IIIa complex, also termed $\alpha_{IIb}\beta_3$ integrin) have become available and are highly effective both in vitro and in vivo in animal models in preventing platelet aggregation and thrombosis [7–9]. Integrins are cell surface heterodimeric glycoprotein receptors that integrate the cytoskeletal activities of a cell with that of its environment, via cell-to-cell and cell-to-extracellular matrix interactions [10]. Indeed, several integrins, including the related vitronectin receptor, $\alpha_v\beta_3$, which are present, for example, on vascular SMCs and platelets, and recognize the Arg–Gly–Asp (RGD) sequence found in many adhesive molecules, participate in the adhesion of vascular SMCs to vitronectin and migration of the cells towards fibronectin, laminin, and collagen types I and IV [10]. Therefore, nonspecific β_3 integrin blocker can block these reactions [11].

Abciximab (Reopro, chimeric 7E3 Fab) is a monoclonal antibody (mAb) fragment that inhibits platelet aggregation induced by physiologic and pathologic agonists by binding to the platelet $\alpha_{IIb}\beta_3$ integrin [12]. This biological activity formed the basis of its development as an antithrombotic agent to prevent and treat platelet-mediated ischemic cardiovascular disease [12]. The primary indication for abciximab is the prevention of ischemic complications in patients undergoing percutaneous coronary revascularization (including percutaneous transluminal coronary angioplasty (PTCA) and atherectomy) [11,12]. Furthermore, the abciximab is crossreactive with other integrin superfamily, including the vitronectin receptors ($\alpha_v\beta_3$) [12]. In vitro, abciximab appears to have similar affinity for the $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins and redistributes between them [13], it is possibly that some effects of abciximab are due to its reactivity with one or the other of these receptors.

The present study was designed to elucidate the detailed mechanisms involved in the inhibition of neointimal hyperplasia by abciximab in a rat model of balloon angioplasty.

1. Materials and Methods

1.1. Materials

Abciximab (Reopro) was a donation from Eli Lilly. EDTA, sodium nitrate, sodium pentobarbital, glutaraldehyde, paraformaldehyde, Tris (hydroxymethyl) aminomethane and trichloracetic acid (TCA) were purchased from Sigma (St. Louis, MO). VCl_3 was obtained from Aldrich (Milwaukee, WI). Antihuman PDGF-BB mAb was purchased from Pepro Tech., England; biotinylated antirabbit IgG and normal goat serum were purchased from Vector Lab. (Burlingame, CA); peroxidase-conjugated streptoavidin was obtained from Dako, Denmark. Cyclic AMP, cyclic GMP, and thromboxane B_2 (TxB_2) EIA kits were purchased from Cayman (Ann Arbor, MI).

1.2. Animals

A total of 62 male Wistar rats weighing 200–250 g were used in all studies. The animals were maintained on a 12-h light/dark cycle under controlled temperature (20 ± 1°C) and humidity (55 ± 5%). Animals were given continuous access to food and water.

1.3. Arterial Injury Model

Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and a 2F embolectomy balloon catheter (Baxter Health Care, Palo Alto, CA) was introduced into the left common carotid artery by way of the external carotid artery. The balloon was inflated with sufficient saline to distend the common carotid and was then withdrawn to the external carotid artery. This procedure was repeated three times. On removal of the catheter, the external carotid artery was ligated, and the wounds were closed. Abciximab or isovolumeric normal saline was infused intravenously at a rate of 0.25 mg/kg/day by the use of osmotic minipumps (Model-2002, Alza, Palo Alto, CA) immediately after the balloon angioplasty, and was continued for 7, 14, and 28 days, respectively, at which time the animals were anesthetized, blood was collected, and the common carotid artery was removed, and stained with hematoxylin–eosin (H&E). The H&E stain was used to visualize the SMCs in the media and intima. Finally, the light microscopic picture was analyzed using a computer-assisted image analyzer, and changes between the neointimal area and the total area within the internal elastic lamina were determined on three cross-sections each. Measurements of each section were made to quantify: (1) neointimal hyperplasia, measured as the increase in vessel wall cross-sectional area, [(media + intima area)/media area], as multiples of area increase [14]; and (2) percentage of the vessel wall lumen occluded [1 − (lumen area/internal elastic lamina area) × 100], as percent occlusion [14]. All measurements were made by an investigator blinded to the treatment and injury conditions.

Blood (5 ml) was collected by aortic puncture in 3.8% sodium citrate (9:1). Citrated blood was immediately centrifuged, and the supernatant was collected and stored at −80°C before cyclic AMP, cyclic GMP, and TxB_2 assays. Nitrate concentrations were determined as described previously [15]. The cyclic AMP, cyclic GMP, and TxB_2 levels were measured with EIA kits.

1.4. Electromicroscopic Examination of Injured Arteries

Electromicroscopic examination of injured arteries after balloon angioplasty was performed according to our previous description [16]. In brief, rats were perfused transcardially with normal saline followed by perfusion with a mixed aldehyde solution. The common carotid artery was removed and postfixed overnight at 4°C in a similar fixative. The artery was trimmed into small cross-sections (≈2 mm) that were further postfixed in 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer solution (pH 7.4) at room temperature for 1.5 h. The selected blocks were then dehydrated in a graded series of alcohol and embedded in an Epon–Araldite mixture (EMS). Ultrathin sections were cut and mounted on 150-mesh copper grids, double-stained with uranyl acetate and lead citrate, and examined under a Hitachi H-600 electron microscope operated at 75 kV.

1.5. Immunohistochemistry of PDGF-BB Expression in Injured Arteries

Immunostaining of PDGF-BB was performed using the streptoavidin–biotin immunoperoxidase method as described previously [17]. In brief, arterial sections were deparafinized in xylene and ethanol baths; endogenous peroxidase activity was quenched in a solution of methanol (200 ml) plus hydrogen peroxide (3%; 50 ml); and nonspecific binding was prevented by preincubation with 10% normal goat serum. Arterial sections were then exposed to the rabbit polyclonal antihuman PDGF-BB IgG (1:100) or rinsed with PBS, and incubated with biotinylated goat antirabbit IgG (1:300). Dot blot and Western blot analyses were performed to confirm the crossreactivity of rabbit polyclonal antibody to rat proteins. Peroxidase labeling was achieved with incubation by use of peroxidase-conjugated streptoavidin (1:300), and antibody visualization was established after a 5-min exposure to 0.05%

3,3′-diaminobenzidine in 0.05 M Tris–HCl, pH 7.6, with 0.003% hydrogen peroxide.

1.6. Statistical Analysis

A single slice was chosen at random from each segment, giving three slices per artery. When areas of each segment were compared (one-way analysis of variance (ANOVA)), no differences between segments were found; therefore, the three slices were averaged to give a single measurement for each carotid artery. The experimental results are expressed as means ± S.E.M. and are accompanied by the number of observations. Data were assessed using ANOVA. If this analysis indicated significant differences among the group means, then each group was compared by the Newman–Keuls method. A value of $P < .05$ was considered statistically significant.

2. Results

2.1. Neointimal Hyperplasia After Balloon Angioplasty in Rats

There were no differences in body weight among the sham-, balloon angioplasty-, and abciximab-

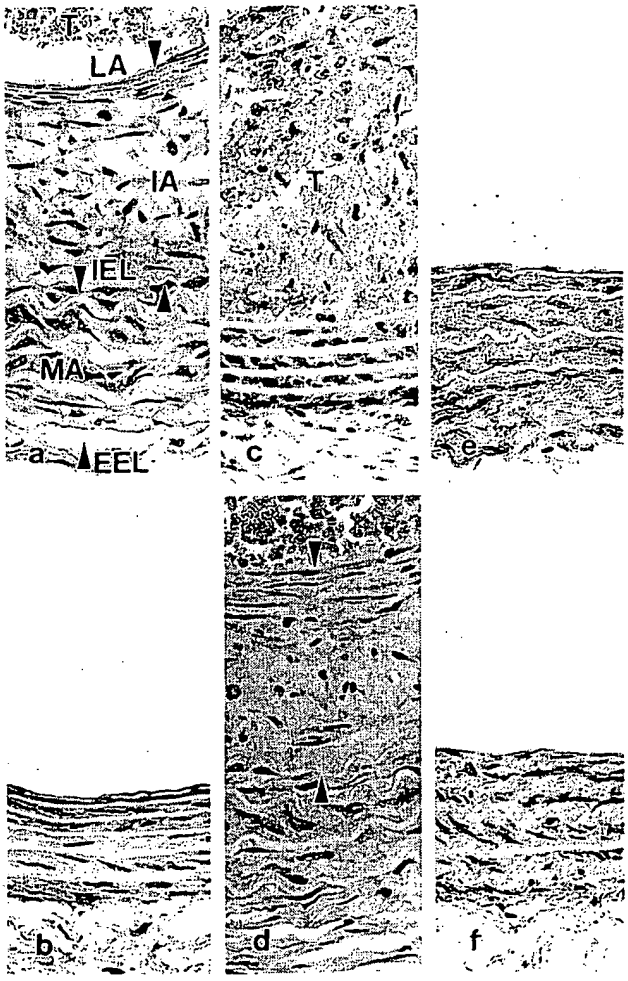


Fig. 1. Microphotographs showing a (a) cross-section of rat carotid arteries 14 days after balloon angioplasty. The intimal area (IA) is formed by the neointima. The medial area (MA) is encircled by the internal elastic lamina (IEL) and external elastic lamina (EEL). Note that the luminal area (LA) is filled with thrombus formation (T). (b) Sham-operated, (c) normal saline treatment for 7 days, (d) 14 days, and abciximab treatment for (e) 7 days and (f) 14 days after balloon angioplasty. Note that obvious neointimal hyperplasia (arrowheads) has developed 14 days after balloon injury.

treated groups, either before surgery or 4 weeks afterward. After 4 weeks of treatment, systolic blood pressure had not significantly changed among these groups (data not shown).

In this study, balloon injury to the carotid arteries in untreated rats occluded the lumen markedly within 7 days after angioplasty (Fig. 1c). The thickness of the media (measured from the external elastic lamina to the internal elastic lamina, see Fig. 1a) of these injured carotid arteries did not differ significantly from media thicknesses of uninjured artery segments (Fig. 1b,c). In contrast, the thickness of the intima of the injured vessel wall segments at 14 days after angioplasty (measured from the internal elastic lamina to the lumen) were markedly thicker than the intima of uninjured arteries (Fig. 1b,d). These data (i.e., the similar overall vessel wall diameters and the similar media thicknesses as compared to the significantly different intimal thicknesses between uninjured and injured vessel walls), plus the histology (Fig. 1), confirm that hyperplasia and subsequent vessel wall occlusion following injuries were due predominantly to intimal SMC proliferation.

The area of neointimal hyperplasia time-dependently increased after angioplasty, which increased about 1.1-fold ($n=8$), 1.9-fold ($n=8$), and 4.4-fold ($n=6$) at 7, 14, and 28 days after arterial injury as compared with sham-operated rats, respectively. Concurrently, neointimal hyperplasia was associated with increases of lumen occlusion from 4.3% (sham-operated rats) to 82.1% within 7 days, 91.0% and 96.1% within 14 and 28 days in injured vessels, respectively ($n=8$). Therefore, neointimal formation determined at 14 days after balloon angioplasty of rat carotid arteries served as controls for all subsequent experiments.

2.2. *Effect of Abciximab on Neointimal Hyperplasia After Balloon Angioplasty*

In a series of experiments, continuous intravenous administration of abciximab to rats with denuded carotid arteries began immediately after angioplasty and continued for up to 14 days. Effective delivery of abciximab by implanted osmotic pumps was maintained throughout the experimental period, because ex vivo ADP-induced platelet aggregation was still inhibited at the end of the observation period (data not shown).

Continuous infusion of abciximab (0.25 mg/kg/day) for 14 days markedly inhibited the neointimal hyperplasia in vessel walls after angioplasty as compared with normal saline-treated rats (Figs. 1f and 2a). This decrease was also associated with a marked decrease in vessel wall occlusion (i.e., about a 87% decrease in vessel wall occlusion) (Fig. 2b). Furthermore, the effects of a continuous 7- and 14-day treatment with abciximab on neointimal hyperplasia and lumen occlusion in carotid arteries after angioplasty were compared (Fig. 2). Fig. 2 shows that abciximab treatment was less effective in preventing neointimal hyperplasia with a period of 7-day infusion than that for 14 days after injury (Fig. 2a). Furthermore, when vessels were continuously treated for 7 and 14 days after injury, respectively, both treatment schedules were equally effective in preventing lumen occlusion (Fig. 2b). These results indicate that integrin ($\alpha_v\beta_3$) inhibition was still needed after the initial period in which

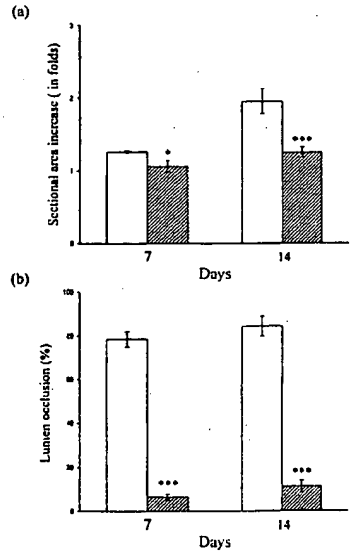
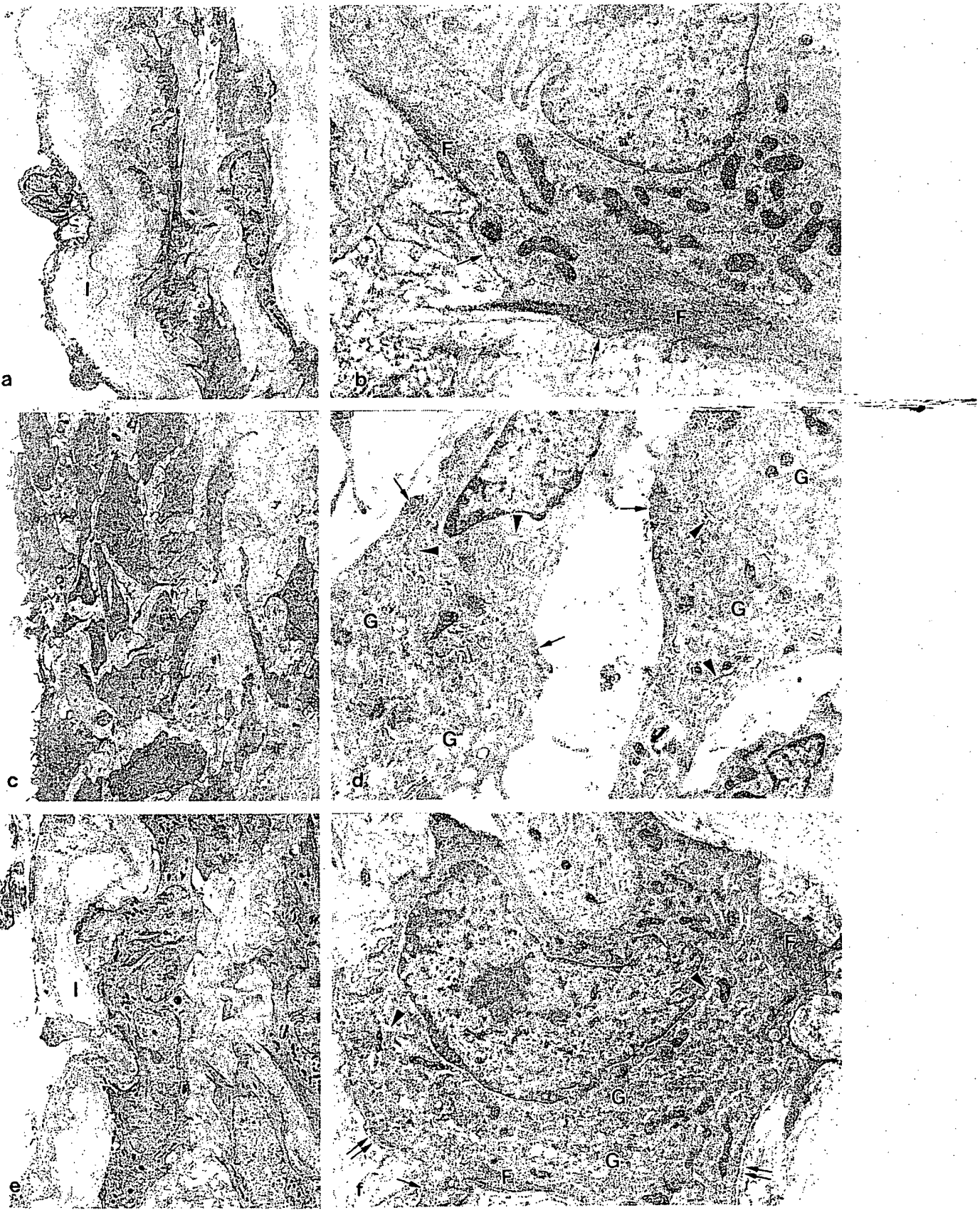


Fig. 2. Inhibitory effects of abciximab on (a) sectional area increase and (b) lumen occlusion after balloon angioplasty of rats. Normal saline (open bars) or abciximab (0.25 mg/kg/day, hatched bars) was infused intravenously for 7 and 14 days after angioplasty of the rat, then neointimal hyperplasia and lumen occlusion of rat carotid arteries were determined as described in Materials and Methods. Data are presented as means ± S.E.M. ($n=8$). * $P<.05$ and *** $P<.001$ compared with normal saline-treated rats.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platelet activation and thrombus formation are believed to occur. At the dose used, no bleeding complications apart from some minor bleeding at the sites of surgery were observed.

2.3. *Effect of Abciximab on Arteries After Angioplasty by Electromicrographic Examinations*

In sham-operated rats, the intima of vessel walls was similar to that of normal rats and composed of a single layer of flattened endothelial cells. Next to the endothelium, there was a thin layer of extracellular matrix with a network of collagen fibrils comprising the major structural component. SMCs alternating with thick elastic lamina in the media displayed numerous processes of variable diameters intermingling with collagen fibrils or bundles (Fig. 3a). The cells showed only a few organelles including a poorly developed endoplasmic reticulum and Golgi complex at each end of or around the nucleus (Fig. 3b). Their cytoplasm was dominated by myofilaments located immediately inside the plasma membrane (Fig. 3b). Seven days after balloon angioplasty, the neointima became multilayered and was occupied by randomly arranged collagen fibers, abundant extracellular matrix and numerous copious cells of irregular shapes (Fig. 3c). Many of the cells had plentiful cytoplasm with an extensive endoplasmic reticulum and numerous Golgi complexes (Fig. 3d). The same cells with a few tiny processes contained very few patches of peripheral myofilaments and lacked a distinct basement membrane (Fig. 3d). With continuous infusion of abciximab 7 days after angioplasty, the intima was composed of a little increased extracellular matrix and collagen fibrils (Fig. 3e). In the media, most of cells contained a prominent endoplasmic reticulum and Golgi complex (Fig. 3f). Interestingly, they also retained typical features of quiescent SMCs, such as a moderate amount of myofilaments, numerous caveolae and the investment by the basement membrane (Fig. 3f).

2.4. *Effect of Abciximab on PDGF-BB Expression by Immunohistochemistry*

The distribution of PDGF-BB within carotid neointima was examined using an antibody that recognizes PDGF-BB but not PDGF-AA or PDGF-AB. From immunohistochemistry, low level of PDGF-BB production was present in the layer of endothelial cells in uninjured arteries (Fig. 4a). Immunoreactivity of PDGF-BB was strongly expressed in thrombus formation and neointimal SMCs, particularly among those located at or near the luminal surface at 7 days after angioplasty (Fig. 4b). The expression of PDGF-BB in neointimal SMCs continued until the end of the experiments (data not shown). The sustained intravenous infusion of abciximab for 7 days significantly reduced PDGF-BB expression in both vessel lumens and neointimal SMCs in arteries after angioplasty (Fig. 4c). This result indicated that abciximab markedly inhibited thrombus formation in carotid arteries after angioplasty, thereby inhibiting the release of PDGF-BB from platelets.

2.5. *Effect of Abciximab on Plasma Nitrate and TxB_2 Formation in Rats After Angioplasty*

As shown in Table 1, nitrate production time-dependently increased at 7 and 14 days after angioplasty in rats. Balloon angioplasty caused

Fig. 3. Ultrastructural changes of vessel walls and smooth muscle cells in (a–b) sham-operated, (c–d) balloon angioplasty, and (e–f) abciximab-treated rats. (a) Only a few collagen fibrils and extracellular matrix between the endothelium and the internal elastic membrane (I) are observed in sham-operated rats which show typical smooth muscle cells bearing numerous processes. (b) Note the characteristic features of the smooth muscle cell, a significant amount of myofilaments (F), a poorly developed endoplasmic reticulum and the caveolae (arrows) along the cytoplasmic membrane. (c) In balloon-lesioned rats, the neointima is full of irregular-shaped cells and extracellular matrix. (d) The irregular-shaped cells possess extensive endoplasmic reticulum (arrowheads), well-developed Golgi complex (G) but very few myofilaments (arrows) under the cytoplasmic membrane. (e) Note a slightly increased amount of collagen fibrils and extracellular matrix in the intima of abciximab-treated rats following angioplasty. (f) The cell in the media shows well-developed Golgi complex (G) and extensive endoplasmic reticulum (arrowheads) but preserves characteristics of typical smooth muscle cell, e.g., abundant myofilaments (F), the caveolae (arrow) and the basement membrane (double arrows).

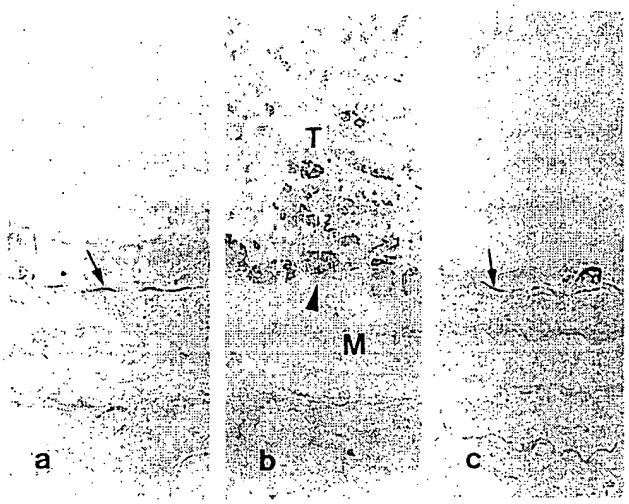

Fig. 4. Microphotographs showing PDGF-BB immunoreactivity in parts of rat carotid arteries 14 days after (a) sham treatment, (b) balloon angioplasty, and (c) balloon injury followed by abciximab treatment. (a) PDGF-BB immunoreactivities are distributed only at the layer of endothelial cells (arrow) in the sham-operated vessels, (b) but evidently at the thrombus formation (T) and its interface with the media (arrowhead) after angioplasty. A similar immunoreactive profile is found between the (a) sham-operated and (c) abciximab-treated vessels (arrow). M, media.

about a 1.5- and 2.1-fold rise in nitrate formation compared with that in sham-operated rats with a 7- and 14-day infusion, respectively (Table 1). Infusion of abciximab (0.25 mg/kg/day) did not significantly change nitrate production in angioplastic rats with a 7- and 14-day infusion, respectively (Table 1). These results imply that the effect of abciximab preventing neointimal hyperplasia was not related to the inhibition of NO production in rats after angioplasty.

On the other hand, plasma TxB_2 concentrations had increased about 10-fold at 7 days after angioplasty and were still elevated at 14 days (Table 1). Abciximab (0.25 mg/kg/day) markedly suppressed the elevation of plasma TxB_2 concentration by about 67% and 69% during continuous the 7- and 14-day infusions, respectively (Table 1).

2.6. *Effect of Abciximab on Cyclic GMP and Cyclic AMP Formation in Rats After Angioplasty*

Table 2 summarizes the time course of cyclic GMP and cyclic AMP levels in plasma after angioplasty and abciximab delivery. The levels of cyclic GMP increased significantly in rats after angioplasty vs. sham-operated rats. How-

Table 1. Effects of abciximab on TxB_2 and nitrate formation in plasma after balloon angioplasty of rats

Group	Nitrate (*M*M)	TxB_2 (ng/ml)
Sham	9.8 ± 1.2	0.27 ± 0.06
Angioplasty		
+SAL (7 days)	15.0 ± 1.3**	2.56 ± 0.15***
+SAL (14 days)	21.3 ± 1.9***	2.97 ± 0.43***
+Abciximab (7 days)	16.5 ± 1.4**	0.84 ± 0.19#
+Abciximab (14 days)	18.7 ± 1.6***	0.93 ± 0.17#

Rats were treated with abciximab (0.25 mg/kg/day) or isovolumeric normal saline (SAL) for 7 and 14 days after balloon angioplasty, then the plasma nitrate and TxB_2 concentrations were determined as described in the Materials and Methods. Data are presented as means ± S.E.M. ($n = 6$).

** $P < .01$.

*** $P < .001$ compared with sham-operated rats.

\# $P < .001$ compared with normal saline-treated rats.

Table 2. Effects of abciximab on cyclic AMP and cyclic GMP formation in plasma after balloon angioplasty of rats

Group	Cyclic GMP (pmol/ml)	Cyclic AMP (pmol/ml)
Sham	9.7 ± 1.3	48.5 ± 7.4
Angioplasty		
+SAL (14 days)	16.5 ± 1.9*	41.8 ± 6.5
+Abciximab (7 days)	15.9 ± 2.5*	54.1 ± 9.1
+Abciximab (14 days)	20.4 ± 3.7*	59.5 ± 8.7

Rats were treated with abciximab (0.25 mg/kg/day) or isovolumeric normal saline (SAL) for 7 and 14 days after balloon angioplasty, then the plasma cyclic AMP and cyclic GMP concentrations were determined as described in the Materials and Methods. Data are presented as means ± S.E.M. ($n = 6$).

* $P < .05$ compared with sham-operated rats.

ever, continuous administration of abciximab (0.25 mg/kg/day) for 7 and 14 days did not significantly change the level of cyclic GMP after angioplasty. Furthermore, no significant differences of cyclic AMP levels were found in plasmas between sham-, balloon angioplasty-, and abciximab-treated rats (Table 2). These results indicate that abciximab inhibition of neointimal hyperplasia was unrelated to the increased levels of cyclic AMP and cyclic GMP in this study.

3. Discussion

Balloon angioplasty disrupts the continuity of the endothelium and induces deep arterial injury, exposing the subendothelium to the blood. The injury sets up key adhesive interactions between cells and their ligands. The circulating platelets can recognize adhesive substrates and rapidly adhere to the damaged surface. The adhesive response of platelets represents an initial step, which may be followed by platelet secretion and aggregation [18]. Activation of platelets is accompanied by secretion of vasoactive substances such as vasoconstrictive agents (TxA_2, serotonin), as well as chemokines and mitogens such as basic fibroblast growth factor (b-FGF) and PDGF, one of the more prominent ones in the formation of neointima. These growth factors stimulate the migration and proliferation of SMCs [19], which are considered to be pivotal in the formation of neointimal hyperplasia [19].

Early clinical trials failed to demonstrate any beneficial effect of antiplatelet agents on restenosis after coronary angioplasty, although the acute complications were significantly reduced [20,21]. There are several potential explanations why these clinical trials have failed to demonstrate the ability of antiplatelet agents to prevent restenosis after angioplasty. First, there are no data to substantiate a reduction of platelet accumulation at the site of injury. Second, even a major reduction in the aggregation response of platelets may be insufficient to prevent substantial release of the chemokines involved in initiation of the proliferative response. Factors released from other cell types (injured endothelial cells, injured medial SMCs, or white blood cells) may play an important role. Third, these antiplatelet agents have negligible effects on platelet adhesion. Thus, the failure of the antiplatelet agents tested in clinical trials should not lead to the conclusion that platelets are not involved in neointimal hyperplasia or that they are an inappropriate therapeutic target.

In this study, we report time-dependent inhibition of neointimal hyperplasia in damaged carotid arteries by an $\alpha_{IIb}\beta_3$ integrin mAb, abciximab. Abciximab can recognize β_3 integrin such as $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ [11,12]. $\alpha_{IIb}\beta_3$ is the platelet receptor for, among others, fibrinogen, and as such is responsible for platelet aggregation and thrombus formation. On the other hand, $\alpha_V\beta_3$ integrin is thought to play a major role in PDGF-stimulated migration of SMCs [22].

In this study, the dose of abciximab employed was 0.25 mg/kg/day, which produced the maximal inhibitory effect on platelet aggregation stimulated by collagen (10 μg/ml) in an ex vivo study (data not shown). Therefore, we used a fixed concentration of abciximab (0.25 mg/kg/day) to characterize its detailed inhibitory mechanisms in neointimal hyperplasia throughout the study. Abciximab time-dependently inhibited both lumen occlusion and neointimal hyperplasia of carotid arteries with continuous infusion for 7 days after angioplasty. However, when abciximab was continually administered for 7 days after injuries, the inhibition of neointimal hyperplasia was less pronounced than that of lumen occlusion (Fig. 2). This discrepancy may result from platelet deposition that occurs immediately after arterial injury, an acute effect (platelet adhesion, activation, and aggregation), which abciximab can effectively inhibit, thereby markedly inhibiting thrombus formation and then reducing lumen occlusion with short-term (7 days) treatment. On the other hand, a shorter treatment period ($\leqq 7$ days) was less effective, indicating that integrin ($\alpha_V\beta_3$) inhibition is still needed beyond the first few days. This late beneficial effect may be related to inhibition of SMC $\alpha_V\beta_3$ receptors. Our preliminary data also revealed that abciximab (0.5 μM) markedly inhibited the adhesion of SMCs to immobilized fibronectin, vitronectin, and laminin (all at 10 μg/ml) at a rate of about 89%, 85%, and 63%, respectively ($n=3$, unpublished data).

PDGF is comprised of two distinct but related polypeptides (A and B) that are products of unique genes [22]. Two separate PDGF receptor genes (α and β) have been identified: $\beta\beta$ receptors bind only PDGF-BB, $\alpha\beta$ receptors bind PDGF-AB or -BB, and $\alpha\alpha$ receptors bind all three isoforms with high affinity [23,24]. Because PDGF released from rat platelets is mostly PDGF-BB [24], SMCs appear able to respond to the rapid appearance of PDGF-BB at sites of vascular injury resulting from platelet degranulation, and it plays an important role in neointimal hyperplasia. Stimulation of SMC chemotaxis and extracellular matrix production are two known properties of PDGF in vitro [22] that seem particularly relevant in this regard. Therefore, we speculated that the obvious effect of abciximab on reduction of PDGF-BB expression in injured vessel after angioplasty may result from inhibition of platelet aggregation associated with secretion, thereby leading to the prevention of PDGF-BB production.

Most experimental models of arterial injuries remove the endothelium, as do balloon angioplasty and surgical endarterectomy in humans, and thus decrease endothelial constitutive NO synthase (cNOS) activity at the injury site [25]. Loss of endothelial NO synthesis, with its ability to inhibit platelet and leukocyte activation and SMC proliferation, appears to play an important role in the restenosis process. In a rat with carotid artery injury, Guo et al. [26] observed that administration of a NO-compound-inhibited neointimal hyperplasia. Interestingly, vascular injury stimulates expression of another NOS isoform, inducible NO synthase (iNOS), in activated SMCs [25]. Hansson et al. [27] detected iNOS gene expression in neointimal but not medial SMCs 1–14 days after balloon-induced rat carotid artery injury. NO production by SMCs may in part compensate for the absence of endothelial NO synthesis by inhibiting SMC proliferation, as well as by limiting thrombus formation by preventing platelet adhesion and aggregation [25,27]. This hypothesis is also supported by our observations, that, in rats, NO concentration markedly increased in plasma after angioplasty (Table 1). Furthermore, in the present study, we show that cyclic GMP formation also increases after angioplasty. This result is in accordance with NO formation: the NO produced is biologically active since most cellular actions of NO occur via stimulation of intracellular guanylate cyclase, leading to an increase in cyclic GMP [28], a potent inhibitor of SMC growth. However, abciximab did not significantly affect the formation of NO or cyclic GMP after angioplasty, indicating that the β_3 integrin mAb attenuating neointimal hyperplasia, at least partly, is not mediated by the NO/cyclic GMP pathway. Furthermore, elevation of cyclic AMP attenuates mitogen-activated protein kinase signaling induced by PDGF in SMCs [29]. However, the results of this study suggest that cyclic AMP might not play an important role in the mediation of neointimal hyperplasia after angioplasty.

TxA_2 is an important mediator of the release reaction and aggregation of platelets [30]. It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation via free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids [31]. In this study, abciximab markedly inhibited TxA_2 formation in plasma after angioplasty in rats. In a rabbit model, the intravenous administration of a thromboxane synthetase inhibitor was effective in reducing platelet deposition after angioplasty [32]. Therefore, it seems likely that abciximab inhibiting TxA_2 formation may play an important role in the inhibition of neointimal hyperplasia after angioplasty.

In conclusion, the most important finding in this study is that abciximab effectively inhibiting neointimal hyperplasia after angioplasty is due, at least partly, to mediation by both the inhibition of TxA_2 formation and PDGF-BB release from platelets, but not by increasing the NO/cyclic GMP or cyclic AMP pathways. The inhibitory properties of abciximab may be involved in the following two mechanisms. (1) Abciximab binds $\alpha_{IIb}\beta_3$ integrin on the platelet membrane in injured arteries after angioplasty, with a resulting inhibition of platelet adhesion, secretion, and aggregation followed by inhibition of TxA_2 formation and PDGF-BB release from platelets, thereby leading to inhibition of lumen occlusion (thrombus formation) and SMC migration, as well as proliferation, and ultimately to inhibition of neointimal hyperplasia. (2) Abciximab may also bind $\alpha_v\beta_3$ integrin on

SMCs, thus subsequently inhibiting cell migration and proliferation, and finally inhibiting neointimal hyperplasia.

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References

1. Gottsauner-Wolf M, Moliterno DJ, Lincoff AM, Topol EJ. Restenosis — an open file. Clin Cardiol 1996;19:347–56.
2. Clowes AW, Clowes MM, Reidy MA. Kinetics of cellular proliferation after artery injury: III. Endothelial and smooth muscle growth in chronically denuded vessels. Lab Invest 1986;54:295–303.
3. Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ. Multiple autocrine growth factors modulate vascular smooth muscle growth response to angiolensin II. J Clin Invest 1993; 91:2268–74.
4. Ellis SG, Bates ER, Schaible T, Weisman HF, Pitt B, Topol EJ. Prospects for the use of antagonists to the platelet glycoprotein Iib/IIIa receptor to prevent postangioplasty restenosis and thrombosis. J Am Coll Cardiol 1991; 17:89B–95.
5. Friedman RJ, Stemerman MB, Wenz B, Moore S, Gauldie J, Gent M, Tiell ML, Spaet TH. The effect of thrombocytopenia on experimental arteriosclerotic lesion formation in rabbits. J Clin Invest 1977;60: 1191–201.
6. Ep JH, Fuster V, Israel D, Badimon L, Badimon J, Chesebro JH. The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J Am Coll Cardiol 1991;17:77B–88.
7. Sheu JR, Teng CM, Huang TF. Triflavin, an RGD-containing antiplatelet peptide, binds to GPIIIa of ADP-stimulated platelets. Biochem Biophys Res Communun 1992;189:1236–42.
8. Sheu JR, Chao SH, Yen MH, Huang TF. In vivo antithrombotic effect of triflavin, an Arg–Gly–Asp-containing peptide on platelet plug formation in mesenteric microvessels of mice. Thromb Haemost 1994;72:617–21.
9. Sheu JR, Yen MH, Peng HC, Chang MC, Huang TF. Triflavin, an Arg–Gly–Asp-containing peptides, prevents platelet plug formation in in vivo experiments. Eur J Pharmacol 1995;294:231–8.
10. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992; 8:929–38.
11. Coller BS. Potential non-glycoprotein Iib/IIIa effects of abciximab. Am Heart J 1999;138: S1–5.
12. Faulds D, Sorkin EM. Abciximab (c7E3 Fab). A review of its pharmacology and therapeutic potential in ischaemic heart disease. Drugs 1994;48:583–98.
13. Le Breton H, Plow EF, Topol EJ. Role of platelets in restenosis after percutaneous coronary revascularization. J Am Coll Cardiol 1996;28:1643–51.
14. Warnke CA, Laio P, Buchanan MR. Quantitative standardization of intimal hyperplasia, using three morphometric measurements. Proceedings of the Microscopical Society of Canada (1993 Book of Abstracts). Toronto: Microscopical Society of Canada, 1993. pp. 172–3.
15. Sheu JR, Hung WC, Kan YC, Lee YM, Yen MH. Mechanisms involved in the antiplatelet activity of *Escherichia coli* lipopolysaccharide in human platelets. Br J Haematol 1998;103: 29–38.
16. Sheu JR, Hung WC, Wu CH, Ma MC, Kan YC, Lin CH, Lin MS, Luk HN, Yen MH. Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia. Circulation 1999; 99:3056–62.
17. Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. Science 1990;248:1009–11.
18. Ruggeri ZM. New insights into the mechanisms of platelet adhesion and aggregation. Semin Hematol 1994;31:229–39.
19. Casscells W. Migration of smooth muscle and endothelial cells. Critical events in restenosis. Circulation 1992;86:723–9.
20. Schwartz L, Bourassa MG, Lesperance J. As-

pirin and dipyridamole in the prevention of restenosis after percutaneous transluminal coronary angioplasty. N Engl J Med 1988; 318:1714–9.
21. Savage M, Goldberg S, Bove AA. Effect of thromboxane A_2 blockade on clinical outcome and restenosis after successful coronary angioplasty: Multi-Hospital Eastern Altantic Restenosis Trial (M-HEART II). Circulation 1995;92:3194–200.
22. Hart CE, Bailey M, Curtis DA, Osborn S, Raines E, Ross R, Forstrom JW. Purification of PDGF-AB and PDGF-BB from human platelet extracts and identification of all three PDGF dimers in human platelets. Biochemistry 1990;29:166–72.
23. Hart CE, Forstrom JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, Bowen-Pope DF. Two classes of PDGF receptor recognize different isoforms of PDGF. Science (Washington, DC) 1988;240:1529–31.
24. Bowen-Pope DF, Hart CE, Seifert RA. Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to different classes of PDGF receptor. J Biol Chem 1989;264:2502–8.
25. Sarkar R, Webb RC. Does nitric oxide regulates smooth muscle cell proliferation. J Vasc Res 1998;35:135–42.
26. Guo JP, Milhoan KA, Tuan RS, Lefer AM. Beneficial effect of SPM-5185, a cysteine-containing nitric oxide donor, in rat carotid artery intimal injury. Circ Res 1994;75:77–84.
27. Hansson GK, Geng YJ, Holm J, Hardharmmar P, Wennmalm A, Jennische E. Arterial smooth muscle cells express nitric oxide synthase in response to endothelial injury. J Exp Med 1994;180:733–8.
28. McDonald LJ, Murad F. Nitric oxide and cyclic GMP signalling. Proc Soc Exp Biol Med 1996;211:1–6.
29. Graves LM, Bornfeldt KE, Raines EW, Potts BC, Macdonald SG, Ross R, Krebs EG. Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. Proc Natl Acad Sci USA 1993;90:10300–4.
30. Hornby EJ. Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A_2 production. Biochem Pharmacol 1982;31:1158–60.
31. Mckean ML, Smith JB, Silver WJ. Formation of lyso-phosphatidylcholine in human platelets in response to thrombin. J Biol Chem 1981;256:1522–4.
32. Faxon DP, Balelli LA, Sandborn T, Haudenschild C, Valeri R, Ryan TJ. The effect of antiplatelet therapy on platelet accumulation after experimental angioplasty in the rabbit iliac model. Int J Cardiol 1992;36:41–7.

both sexes, i.e. reduced VLDL+ILDL 30 to 50%, reduced LDL 15 to 20% and increased HDL 20 to 40% (p's<0.05). E2 potentiated soy's VLDL+ILDL-lowering effects and antagonized soy's HDL-elevating effects. However, E2 and soy had no consistent interactive effects on atherosclerosis. These findings confirm that E2 has significant independent atheroinhibitory activity but indicate no interactive effects of E2 and soy protein or PE. Furthermore, they indicate that both soy protein and PE have atheroinhibitory properties but that responses to each may differ by sex.

The Synthetic Estrogen Receptor Modulator Raloxifene Hydrochloride is Not Vasoprotective in the Rat Carotid Injury Model 1738

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Background: Previous studies from our laboratory have shown that 17β-estradiol (E2) inhibits both in vivo adventitial activation following endoluminal vascular injury and in vitro adventitial fibroblast migration via an estrogen receptor (ER) dependent pathway, suggesting a novel mechanism of E2-mediated vasoprotection. The current study tested the effects of the selective ER modulator raloxifene hydrochloride (Ral) on fibroblast migration in vitro and on neointima formation in balloon-injured rat carotid arteries. **Methods and results:** Primary cultures of adventitial fibroblasts from female Sprague-Dawley(SD) rats were transduced with retroviral particles (pLBg) coordinating the expression of lacZ. Fibroblast migration was analyzed by a Boyden chamber-type assay. Treatment of fibroblasts with vascular smooth muscle cell conditioned media stimulated a two-fold increase in fibroblast migration that was inhibited by E2 (10^{-9} to 10^{-7}M). Ral (10^{-7} to 10^{-6}M) did not attenuate fibroblast migration. Ovariectomized SD rats were treated with Ral (3mg/kg/d or 750 μg/kg/d) or vehicle (Veh) 72 hrs prior to carotid artery balloon injury and continuing throughout the study. Intima/media ratios at 14 days post injury were: Veh 98±4%; Ral (3mg/kg/d) 87±8%; and Ral (750μg/kg/d) 81±7%. Thus, neointima formation did not differ between experimental groups. This study suggests that Ral lacks ER-dependent vasoprotective effects in the rat carotid injury model.

Differential Inhibition of Vascular Cell Adhesion Molecule (VCAM)-1 Expression by Sex Steroid Hormones Mediated by Selective Inhibition of NF-κB, AP-1 and GATA Transcription Factors in Human Vascular Endothelial Cells 1739

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Clinical studies suggest beneficial effects of hormonal replacement therapy in cardiovascular disease. Since atherosclerosis is an inflammatory process involving the induction of cytokines and cellular adhesion molecules, we postulate that estrogens may attenuate atherogenesis by decreasing the expression of adhesion molecules. We investigated the effects of 17β-estradiol (E_2), progesterone (P) and testosterone (T) on human endothelial cell vascular cell adhesion molecule-1 (VCAM-1) expression and characterized the molecular pathways involved. In a concentration-dependent manner, both E_2 and P decreased bacterial lypopolisaccharide (LPS)-induced endothelial cell VCAM-1 mRNA and protein expression, with E_2 being significantly more potent than P at equimolar concentrations. This decrease was associated with a marked decrease in monocytoid cells adhesion to endothelial monolayers. In contrast, T did not exert any significant effect on VCAM-1 expression and monocyte adhesion. E_2 treatment was associated with a complete inhibition of LPS-induced activation of nuclear factor-κB (NF-κB) by gel-shift assay, Western blotting, and immunofluorescence analysis, and to a lesser extent, activator protein-1 (AP-1) and GATA activation. P decreased only AP-1 activation, but to a lesser extent than E_2, while T had no effect on the three transcription factors. By transiently transfecting deletional VCAM-1 promoter constructs into bovine endothelial cells, we found that E_2 inhibited more effectively than P LPS-induced VCAM-1 gene transcription with promoter constructs containing NF-κB, AP-1 and GATA cis-acting elements, while only E_2but not P, was effective on a deletional promoter containing only the κB sites. T had no effect on any of the promoter constructs. These data demonstrate that estrogens and progestins, but not androgens, differentially regulate VCAM-1 expression through distinct signaling pathways and suggest that these agents may be useful in the treatment of atherosclerotic or vascular inflammatory diseases.

Effects of Estrogen and Raloxifene on Markers of Vascular Inflammation in Postmenopausal Women 1740

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Markers of inflammation, including interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1), are associated with increased risk of cardiovascular events in apparently healthy populations. We and others have shown previously that estrogen reduces levels of cellular adhesion molecules including ICAM-1, E-selectin, and vascular cell adhesion molecule (VCAM-1) in postmenopausal women (PMW). Because of increasing interest in the use of selective estrogen receptor modulators in order to avoid cancer risks associated with estrogen therapy, we compared the effects of conjugated equine estrogens (CEE) 0.625 mg, raloxifene 60 mg, or placebo daily, each for 1 month in a double-blind, randomized, double crossover trial with 23 healthy PMW. Nonsignificant increases in IL-6 were seen with CEE and raloxifene therapies. CEE significantly reduced levels of E-selectin, ICAM-1, and VCAM-1. Raloxifene significantly reduced levels of E-selectin and ICAM-1, but had no effect on VCAM-1. The raloxifene lowers levels of cell adhesion molecules, but to a lesser extent than estrogen otherwise healthy PMW. The biological relevance of these changes in markers of inflammation in particular reduction in cell adhesion molecules despite slight increases in IL-6, remains be determined in future vascular studies.

	IL-6 (pg/mL)	E-selectin (ng/mL)	ICAM-1 (ng/mL)	VCAM-1 (ng/mL)
Placebo	2.0±1.3	43.7±13.3	201±40	547±154
CEE	2.3±1.1	38.6±11.3*†	190±38**	503±130*†
Raloxifene	2.4±1.1	41.1±13.0**	196±41*	545±166

Data=mean±SD. *=P(0.05, **=P(0.01 vs. Placebo †=P(0.05 vs raloxifene

Vascular Effects of Estrogen Therapy in Postmenopausal Women with Coronary Artery Disease on Conventional Medical Management 174

Diane Zanger, Arnon Blum, Londa Hathaway, Rita Mincemoyer, Julio A Panza, William H Schenke, Richard O Cannon III, NIH, Bethesda, MD

Estrogen (E) has been shown to improve arterial endothelium-dependent vasodilation and reduce vascular markers of inflammation in healthy postmenopausal women (PMW). To determine whether E also has nitric oxide (NO)-dependent vascular effects in PMW with chronic stable coronary artery disease (CAD), we measured serum nitrogen oxides (chemiluminescence technique) on a nitrate restricted diet (as an index of endothelial NO release), flow-mediated brachial artery dilation (FMD) by ultrasound following forearm ischemia (as an index of NO bioactivity), and cell adhesion molecule levels [E-selectin, intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1)] in serum (as an index of NO-regulated inflammatory markers) in 10 CAD PMW on medical management (including statin lipid-lowering drugs-8). PMW were randomly assigned to conjugated equine E (CEE) 0.625 mg or placebo daily for 1 month, with crossover to the alternate therapy after 1 month off therapy in a double-blind study. CEE did not increase indices of NO release or bioactivity relative to placebo treatment, but significantly lowered levels of cell adhesion molecules. Thus, E does not improve endothelium- dependent vasodilator responsiveness in PMW with CAD on conventional medical management, but does reduce levels of cell adhesion molecules by a mechanism likely independent of NO.

	NO (μmol/L)	FMD (%)	E-selectin (ng/mL)	ICAM-1 (ng/mL)	VCAM-1 (ng/mL)
Placebo	34.9±5.1	7.6±2.2	56.3±6.9	303±26	657±71
CEE	31.2±4.6	8.1±2.6	46.9±6.1**	282±25*	605±73**

Data=mean±SEM
*=P(0.05, **= P(0.01 vs placebo

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Abstracts 1742–1751

Identification of a Urokinase Receptor-Integrin Interaction Site: Promiscuous Regulator of Integrin Function 1742

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Background: The integrin family of adhesion receptors is involved in inflammation, hemostasis, angiogenesis, and tumor metastasis. Adhesion and signaling by integrins require their dynamic association with non-integrin membrane proteins. One such protein, the glycolipid-anchored urokinase receptor (uPAR), associates with and modifies the function of the β2 integrin Mac-1 (CD11b/CD18). However, the site(s) of interaction between uPAR and Mac-1 is unknown. **Results:** In this study a critical non-I domain binding site for uPAR on CD11b (M25) is identified. Recombinant soluble uPAR and cells expressing uPAR bound to immobilized M25, binding being promoted by urokinase and blocked by soluble M25 (IC50=2 μM), but not by a scrambled control or homologous peptides from other β2-associated αsubunits (i.e., CD11a and CD11c). In the β-propeller model of αsubunit folding, M25 spans an exposed loop on the ligand-binding, upper surface of CD11b, thereby identifying uPAR as an atypical CD11b ligand. Although not blocking ligand binding to Mac-1, M25 (25-100 μM) inhibited leukocyte adhesion to fibrinogen, vitronectin, and cytokine-stimulated endothelial cells. M25 also blocked the association of uPAR with β1 integrins, as determined by co-immunoprecipitation techniques, and impaired β1 integrin-dependent spreading and migration of human vascular smooth

muscle cells on fibronectin and collagen. Conclusion: In this study we have identified a non-l comsin-peptide-sequence, M25, within the αsubunit of Mac-1 that critically regulates the binding of Mac-1 to the non-integrin receptor uPAR. To our knowledge, the M25 peptide sequence is the first extracellular domain sequence of an integrin which broadly impairs integrin adhesion and migration to matrix proteins without directly inhibiting overall ligand binding, suggesting a novel strategy for regulation of integrin function in vascular injury and inflammation associated with atherosclerosis and restenosis.

1743 Role of CD11/CD18 Integrins in Leukocyte Trafficking in the Carotid Artery

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Leukocyte infiltration plays a crucial role not only in acute and chronic vascular injuries but also in lesion repair and vascular remodeling. To understand better the molecular mechanisms that regulate leukocyte trafficking in the arterial wall, we developed a vascular injury model in the mouse carotid artery by perivascular application of cupric oxide, a potent oxidative stimulus that induces tissue inflammation, for study in knockout mice. This model exhibits consistent leukocyte infiltration without any mechanical disruption of vascular endothelium, which is problematic in some perivascular injury models. Leukocytes infiltrate into the vascular wall primarily from the adventitia and also from the lumen side within the first 24 h. At 48 h after application of cupric oxide, numerous leukocytes are observed in the adventitia, media and intima. At 72 h after application of cupric oxide, massive leukocyte infiltration is observed in each layer of the vascular wall with multiple focal necrosis. To investigate the effects of CD11/CD18 integrins on leukocyte trafficking in vascular injury, mice deficient in CD11a, CD11b and CD18 were compared with wild type mice in cupric oxide-induced carotid vasculitis. At 48 h after application of cupric oxide, the numbers of leukocytes in the intima, media and adventitia of carotid arteries of mice deficient in CD11a ($n=5$) and CD11b ($n=5$) were similar to that of wild type mice ($n=5$). Mice deficient in CD18 ($n=3$), which therefore lack both CD11a and CD11b, had numerous leukocytes present in the adventitia, but leukocytes were completely absent in the media and intima of the carotid. Conclusion: CD18 is required for leukocyte trafficking into the intima and media of the carotid artery post cupric oxide injury, whereas a deficiency of CD11a or CD11b alone did not alter leukocyte trafficking.

1744 Relative Contribution of LFA-1 and Mac-1 to Neutrophil Adhesion and Migration

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To define better the unique and overlapping functions of CD11a and CD11b, mice deficient in CD11a were developed and neutrophil function was compared both *in vitro* and *in vivo* with mice deficient in CD11b, mice deficient in CD18 which therefore lack both CD11a and CD11b, and wild type mice. Although mice deficient in CD11a have a peripheral leukocytosis similar to mice deficient in CD18, they do not develop spontaneous infections, in marked contrast to the high incidence of infections observed in mice deficient in CD18. As expected, the adhesion of CD18-deficient neutrophils to either intercellular adhesion molecule 1 (ICAM-1) or murine endothelial cells did not increase after zymosan-activated serum (ZAS) stimulation. CD11a-deficient neutrophils demonstrated activation by ZAS as evidenced by upregulation of CD11b on the cell surface, but did not show increased adhesion to either purified ICAM-1 or endothelial cells. Adhesion of CD11b-deficient neutrophils was significantly increased with ZAS stimulation, although adhesion was lower than for wild type neutrophils. The strength of adhesion through CD11a, CD11b, and CD18 was evaluated by the ability of neutrophils to maintain firm attachment with increasing shear stress. CD11a-deficient neutrophils showed a marked reduction in adhesion with increased detachment of neutrophils similar to CD18-deficient neutrophils, with only a modest but significant reduction caused by an absence of CD11b. Leukocyte influx in a subcutaneous air pouch in response to TNF-α was significantly reduced by 60-70% in CD11a- or CD18-deficient mice ($p<0.01$), whereas leukocyte influx in CD11b-deficient mice was significantly increased by more than 100% ($p<0.01$). Summary: Genetic deficiencies in CD11a and CD11b clearly demonstrate the unique contribution of each molecule to both neutrophil adhesion and extravasation.

1745 VLA-4-Engagement Activates Monocyte Adhesion via LFA-1 and Mac-1: Control by the Urokinase-Receptor

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β_1- and β_2-integrins mediate leukocyte adhesion and migration through the vessel wall in a cascade-like fashion during acute inflammatory processes. We studied the effect of VLA-4 ($\alpha_4\beta_1$) engagement on monocytes on β_2-integrin mediated adhesion to cultured human endothelial cells (HUVEC) and the role of the urokinase receptor (uPAR) as a regulator of β_2-integrin function in this setting. Ligation of VLA-4 by its natural ligand soluble VCAM-1-IgG induced 3-fold adhesion to HUVEC. In addition, two monoclonal antibodies (mAb) against the β_1-integrin chain of VLA-4 were identified, which induced monocyte adhesion to the immobilized ligands of β_2-integrins fibrinogen und ICAM-1 (2-3 fold, each), as well as to ICAM-1 transfected CHO-cells (3-4 fold) and HUVEC (6-8 fold). Induced adhesion was blocked in the presence of mAbs anti-CD18 (70-90%), anti-CD11a (50-60%, except adhesion to specific CD11b-ligand fibrinogen) or CD11b (60-70%). Induced adhesion required the presence of uPAR, since the removal of uPAR by phosphatidyl-inositol-specific phospholipase C diminished adhesion by 64%+6.9 (mean+SEM). After VCAM-1-IgG binding, FACS-analysis showed increased monocyte surface expression of the activation-dependent epitope CBRM1/5 on CD11b without quantitative changes for CD11a, CD11b or CD18. VLA-4 ligation by VCAM-1-IgG or mAbs did not result in direct VLA-4 activation, since adhesion to fibronectin o VCAM-1 were not induced, and induced adhesion to HUVEC was not inhibited in the presence of blocking mAbs anti-VLA-4. Conclusion: Engagement of VLA-4 triggers rapid activation of the β2-integrins Mac-1 und LFA-1 and thereby enhanced adhesiveness, which is controlled by the urokinase receptor. These mechanisms may play a role during acute inflammatory processes during ischemia and reperfusion.

1746 Platelet Glycoprotein Ibα Is an Adhesive Ligand for the Leukocyte Integrin Mac-1 (CD11b/CD18)

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Leukocyte-platelet complexes participate in vascular inflammation associated with plaque activation and restenosis. Previous studies have shown that Mac-1, the primary fibrin(ogen) receptor on leukocytes, directly facilitates the recruitment of leukocytes at sites of platelet and fibrin deposition. While Mac-1 may interact with fibrinogen bound to platelet GP IIb/IIIa or possibly to ICAM-2, the precise Mac-1-dependent platelet counter-ligand is unknown. Given the homology between the ligand-binding vWF A1 domain and the Mac-1 I domain, we hypothesized that Mac-1 would bind the platelet vWF receptor GP Ibα. We now report a direct interaction between Mac-1 and GP Ibα. THP-1 monocytic cells and transfected cells that express Mac-1 bound to purified GP Ibα-coated wells. Inhibition studies with mAbs or receptor ligands (Table I) suggested that: (1) the I domain is a recognition site on Mac-1 for GP Ibα, and (2) the leucine-rich repeat and flanking regions of GP Ibamediate Mac-1 binding. Neutrophils from wild-type but not Mac-1-deficient mice bound to GP Ibα, establishing the specificity of the interaction between Mac-1 and GP Ibα. Thus, these observations indicate that GP Ibais an adhesive ligand for Mac-1 and provide a molecular strategy for disrupting leukocyte-platelet complexes that promote vascular inflammation in atherosclerosis and restenosis.

Adhesion of Cytokine-Stimulated THP-1 Cells to GP Iba

MAb or Ligand	Receptor Target	% Inhibition ($p<0.01$)
IB4	anti-CD18	99 ± 01*
TS1/22	anti-CD11a	10 ± 13
LPM19c	anti-CD11b	92 ± 12*
VM16d	anti-GP Iba	83 ± 16*
Fibrinogen	Mac-1	99 ± 01*
vWF A1 Frag	GP Iba	83 ± 13*

1747 Abciximab Binds to the Leukocyte Integrin Mac-1 (CD11b/CD18, $\alpha_M\beta_2$) and thereby Results in a Functional Blockade *In Vitro* and *In Vivo*

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Besides the blockade of the integrin GP IIb/IIIa the antibody-fragment abciximab (ReoPro®, c7E3) may provide clinical benefits by its cross-reactivity and thus blockade of other integrin receptors. We evaluated whether binding of abciximab to the leukocyte integrin Mac-1 has functional consequences and is present at therapeutic concentrations in patients. In vitro binding of fluorescence labeled abciximab to monocytic THP-1 cells, monocytes and granulocytes could be detected in flow cytometry and was inhibited by a Mac-1 blocking monoclonal antibody (mAb) clone 44. The binding of the Mac-1 ligands fg, Factor X and iC3b, evaluated by flow cytometry, could be inhibited by coincubation with abciximab (10μg/ml). As a functional consequence the conversion of factor X to factor Xa mediated by THP-1 cells (1.06±0.09 U/ml), as detected by the chromogenic factor Xa substrate SZ-2211, was inhibited by abciximab (0.36±0.02 U/ml, $p<0.001$) to the same extent as by the Mac-1 blocking mAb (0.28±0.05, $p<0.001$). Adhesion of THP-1 cells to immobilized ICAM-1, that mediates leukocyte adhesion on endothelium, was reduced to 52±8% ($p<0.001$) and to fg to 23.3±4% ($p<0.001$) by abciximab. Fg mediated THP-1-Aggregation was also clearly impaired. Abciximab binding to stimulated monocytes of four patients could be detected during abciximab administration (bolus and 12h infusion). Subsequently it decreased gradually during the following 5 days (mean fluorescence 0h:23±10, 10min:213±132, 5d:52±16). In the same time fg binding to monocytes, which was significantly reduced during abciximab infusion, recovered slowly (0h:2238±631, 10min:307±115, 120h:1678±645). This blockade of Mac-1 by abciximab could provide additional clinical benefits beyond the well-described blockade of GP IIb/IIIa.

1748 Reduced Neointima Hyperplasia of Vein Grafts in ICAM-1-deficient Mice

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Recently, we have established a new mouse model of vein graft arteriosclerosis by grafting vena cava to carotid arteries. In many respects, the morphological features of this murine vascular graft model resemble those of human venous bypass graft disease. Using this model, we studied the role of intercellular adhesion molecule-1 (ICAM-1) in the development of vein graft arteriosclerosis in ICAM-1-deficient mice. Neointimal hyperplasia of vein grafts in ICAM-1 -/- mice were reduced 30% to 50% compared to wildtype controls. Immunofluorescent analysis revealed that increased ICAM-1 expression was observed on the endothelium

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between the ascending aorta and the pulmonary artery, requiring surgery to correct.

a. stenosis Narrowing of the aorta or its orifice due to lesions of the wall with scar formation. It is caused by infection, as in rheumatic fever, or by embryonic anomalies. Hypertrophy of the heart is a common result. SYN: *aortostenosis*. SEE: *Nursing Diagnoses Appendix*.

SYMPTOMS: Symptoms include dyspnea on exertion, fatigue, exertional syncope, angina, and palpitations. Orthopnea and paroxysmal nocturnal dyspnea are due to left ventricular failure. Peripheral edema may be present. Palpation detects diminished carotid pulses and pulsus alternans, displacement of the apical impulse, and a systolic thrill at the base of the heart, at the jugular notch, and along the carotid arteries.

NURSING IMPLICATIONS: The nurse obtains a history of related cardiac disorders. Cardiopulmonary function is assessed regularly by monitoring vital signs and weight, intake, and output for signs of fluid overload. The patient is monitored for chest pain, which may indicate cardiac ischemia, and the electrocardiogram evaluated for ischemic changes. Activity tolerance and fatigue are assessed.

After cardiac catheterization, the insertion site is checked according to protocol (often every 15 min for 6 hr) for signs of bleeding; the patient is assessed for chest pain, and vital signs, heart rhythm, and peripheral pulses distal to the insertion site are monitored. Problems are reported to the cardiologist.

Desired outcomes include adequate cardiopulmonary tissue perfusion and cardiac output, reduced fatigue with exertion, absence of fluid volume excess, and ability to manage the treatment regimen.

a. valve The valve between the left ventricle and the ascending aorta that prevents regurgitation of blood back into the left ventricle. It is composed of three segments, each of which is called a semilunar cusp. The valve is open during ventricular systole and closed during diastole.

aortitis (ā-or-tī'tĭs) [L. from Gr. *aorte*, aorta, + *itis*, inflammation] Inflammation of the aorta. This condition is associated with syphilis and other diseases in which vascular changes have taken place. It is a common cause of aortic aneurysm. Symptoms include possible cough, cyanosis, dyspnea, cardiac asthmatic attacks, and hemoptysis.

aortoclasia (ā"or-tō-klā'zē-ă) [" + *klasis*, a breaking] Aortic rupture.

aortocoronary (ā-or"tō-kor'ŏ-nā-rē) Pert. to both the aorta and the coronary arteries.

aortocoronary bypass Coronary artery bypass surgery.

aortogram (ā-or'tō-grăm") [" + *gramma*, something written] An image of the aorta obtained through radiography, computed tomography, or magnetic resonance imaging, usually after the injection of a contrast agent.

aortography (ā"or-tog'ră-fē) [L. from *aorte*, aorta, + *graphein*, to write] Radiography of the aorta after injection of a contrast medium. **aortographic** (ā"or-tō-grăf'ĭk) *adj*.

retrograde a. Aortography by injection of a contrast medium into the aorta or one of its branches, and thus against the direction of the blood flow.

translumbar a. Aortography by injection of a contrast medium into the abdominal aorta through a needle inserted in the lumbar area near the level of the last rib.

aortoiliac (ā-or"tō-ĭl'ē-ăk) Pert. to both the aorta and the iliac arteries.

aortolith (ă-or'tō-lĭth) [" + *lithos*, stone] A calcareous deposit in the aortic wall.

aortomalacia (ā-or"tō-mă-lā'shē-ă) [" + *malakia*, softness] Softening of the wall of the aorta.

aortoplasty (ā-or"tō-plăs"tē) Surgical repair of the aorta.

aortorrhaphy (ā"or-tor'ă-fē) [" + *rhaphe*, seam, ridge] Suture of the aorta.

aortosclerosis (ā-or"tō-sklĕr-ō'sĭs) [" + *sklerosis*, hard] Aortic sclerosis.

aortostenosis (ā-or"tō-stĕ-nō'sĭs) Aortic stenosis.

aortotomy (ā"or-tŏt'ō-mē) [" + *tome*, cutting] Incision of the aorta.

AOS *anodal opening sound*.

A.O.S.S.M. *American Orthopedic Society for Sports Medicine*.

A.O.T.A. *American Occupational Therapy Association*.

A.O.T.F. *American Occupational Therapy Foundation*.

A.P. *anteroposterior*.

A.P.A. *American Pharmaceutical Association; American Physiotherapy Association; American Podiatry Association; American Psychiatric Association; American Psychological Association*.

APACHE II Acronym for *Acute Physiology and Chronic Health Evaluation*, a severity of disease classification system.

apallesthesia (ă-păl"ĕs-thē'zē-ă) [" + *lein*, to tremble, + *aisthesis*, sense] Inability to sense vibrations. SEE: *pathesia*.

apancreatic (ă-păn"krē-ăt'ĭk) **1.** Caused by absence of the pancreas. **2.** Pert. to or involvement of the pancreas.

aparalytic (ă-păr"ă-lĭt'ĭk) [Gr. *a-*, + *paralyein*, to loosen] Marked by lack of paralysis.

aparathyrosis (ă-păr"ă-thī-rō'sĭs) [" + *para*, near, + *thyreos*, an oblong shield, + *osis*, condition] Parathyroid deficiency.

apareunia (ă"păr-ū'nē-ă) [" + *pareunos*, going with] Inability to accomplish sexual intercourse. SEE: *dyspareunia*.

aparthrosis (ăp"ăr-thrō'sĭs) [Gr. *apo-*, + *arthron*, joint, + *osis*, condition] A joint that moves freely in any direction

such as the shoulder joint. SYN: *diarthrosis*. **2.** Dislocation of a joint.

apathetic (ăp"ă-thĕt'ĭk) [" + *pathos*, disease, suffering] Indifferent; without interest. SYN: *apathic*.

apathic (ă-păth'ĭk) Apathetic.

apathism (ăp'ă-thĭzm) [" + *pathos*, disease, suffering, + *ismos*, condition] Slowness to react to stimuli; opposite of erethism.

apathy (ăp'ă-thē) [Gr. *apatheia*] Indifference; insensibility; lack of emotion. SYN: *[illegible]*

apatite (ăp'ă-tīt") [Ger. *Apatit*, "the deceptive stone"] A mineral containing calcium and phosphate ions and a univalent anion in a specific ratio; the major constituent of teeth and bones.

APC *absolute phagocyte count*.

A.P.C *aspirin, phenacetin, and caffeine*, common ingredients in various headache and cold tablets. Phenacetin is no longer considered suitable for use in any form.

APF *anterior pituitary extract*.

apellous (ă-pĕl'ŭs) [Gr. *a-*, not, + L. *pellis*, skin] **1.** Lacking skin. **2.** Lacking foreskin; circumcised.

apepsia (ă-pĕp'sē-ă) [Gr. *a-*, not, + *pepsis*, digesting] Cessation of digestion.

apepsinia (ă"pĕp-sĭn'ē-ă) Absence of pepsin in the gastric juice.

aperient (ă-pēr'ē-ĕnt) [L. *aperiens*, opening] **1.** Having a mild laxative effect. **2.** A mild laxative.

aperiodic Occurring other than periodically.

aperistalsis (ă"pĕr-ĭ-stăl'sĭs) [Gr. *a-*, not, + *peri*, around, + *stalsis*, constriction] Absence of peristalsis.

aperitif (ă-pĕr"ĭ-tēf') [L. *aperire*, to open] An alcoholic beverage, such as wine, taken before a meal to stimulate the appetite.

aperitive (ă-pĕr'ĭ-tĭv) **1.** Stimulating the appetite. **2.** Aperient.

Apert's syndrome (ă-pārz') [Eugene Apert, Fr. pediatrician, 1868–1940] A congenital condition marked by a peaked head and webbed fingers and toes. Oral manifestations include cleft palate or uvula, a prognathic mandible, and maxillary hypoplasia resulting in extreme malocclusion.

apertura (ăp"ĕr-tū'ră) *pl.* **aperturae** [L.] An opening.

aperture (ăp'ĕr-chūr") An orifice or opening, esp. to anatomical or bony spaces or canals.

apex (ā'pĕks) *pl.* **apexes, apices** [L., tip] The pointed extremity of a conical structure.

apical (ăp'ĭ-kal, ā'pĭ-kal), *adj*.

a. of the lung The superior, subclavicular portion of the lung.

root a. The end of the root of a tooth.

apex beat In systole, the movement of the apex of the left ventricle against the chest wall. It is felt in the fifth left intercostal space, approx. 3 ½ in. (8.9 cm) from the middle of the sternum, about 1 in. (2.5 cm) within a line drawn down from the middle of the clavicle parallel with the sternum (the mammary line). Generally it may be detected by inspection or palpation; when

these fail, [illegible] tation. As a amined in while breat lying down. 1 in. (2.5 c body is incl be detecte mary line. may becom low its usu ration, the and may be

ABNORM may be not and in hea emphysem

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apexcardiogra wall moven beat.

apexigraph, a grăf') [L. *a* write] An in location an root.

apex murmur heart.

Apgar score [siologist, 19 uating an i birth. The i muscle ton color are ra min, after b 1, or 2; the r *terpretation*

tion of tissue. Histologically, it involves the dilatation and increased permeability of small blood vessels, which result in migration and accumulation of leukocytes and exudation of plasma proteins into the area. The result dilutes, destroys, or walls off the injurious agent and injured tissue and is marked by the classic signs of inflammation: pain (dolor), heat (calor), redness (rubor), swelling (tumor), and loss of function (functio laesa). SEE: *leukotrienes.*

physiological stress r. Stress r.

reticulocyte r. An increase in reticulocyte production in response to the administration of a hematinic agent.

stress r. The predictable physiological response that occurs in humans as a result of injury, surgery, shock, ischemia, or sepsis. SYN: *physiological stress r.*

This response is hormonally mediated and is divided into three distinct phases:

Ebb phase (lag phase): For 12 to 36 hr after the precipitating event, the body attempts to conserve its resources. Vital signs (heart, respiration, temperature) are less than normal. *Flow phase (hypermetabolic phase):* This stage peaks in 3 to 4 days and lasts 9 to 14 days, depending on the extent of the injury or infection and the person's physical and nutritional status. Carbohydrate, protein, and fat are mobilized from tissue stores and catabolized to meet the energy needs of an increased metabolic rate (hypermetabolism). Serum levels of glucose and electrolytes such as potassium can increase dramatically. If this stage is not controlled by removal of the cause or activator, multiple system organ failure or death can result. *Anabolic phase (recovery):* The anabolic, or healing, phase occurs as the catabolism declines, and electrolyte balances are restored. Often, aggressive nutritional support is necessary to promote a positive nitrogen balance.

triple r. The three phases of vasomotor reactions that occur when a pointed instrument is drawn across the skin. In order of appearance, these are red reaction, flare or spreading flush, and wheal.

unconditioned r. An inherent response rather than one that is learned. SEE: *reflex, conditioned.*

rest (rĕst) [AS. *raest*] **1.** Repose of the body caused by sleep. **2.** Freedom from activity, as of mind or body. **3.** To lie down; to cease voluntary motion. **4.** A remnant of embryonic tissue that persists in the adult.

restenosis (rĕ″stĕ-nō′sĭs) [L. *re,* again, + Gr. *stenos,* narrow] The recurrence of a stenotic condition as in a heart valve or vessel.

restiform (rĕs′tĭ-form) [L. *restis,* rope, + *forma,* shape] Ropelike; rope-shaped.

resting Inactive, motionless, at rest.

resting cell **1.** A cell not in the process of dividing. SEE: *interphase.* **2.** A cell that is not performing its normal [illegible] nerve cell that is not conducting a pulse or a muscle cell that is not contracting).

resting pan splint Splint designed to maintain fingers and stabilize hand in functional position with the fingers in position. Also called *resting hand splint.*

restitutio ad integrum (ĕs-tĭ-tū′shē-ō ăd ĭn′tĕ-grŭm) [L.] Complete recovery to health.

restitution (rĕs″tĭ-tū′shŭn) [L. *restitutio*] **1.** The return to a former position; the act of making amends. **2.** The turning of a fetal head to the right or left after it has completely emerged through the birth canal.

restless legs syndrome A condition of unknown etiology marked by [illegible] creeping and internal [illegible] occurring in the lower extremities, causing an almost irresistible urge to move the legs. The symptoms are worse at the end of the day when the person is either seated or in bed. This disorder is sometimes associated with the [illegible] nal colic caused by the attempted the actual passage of a renal [illegible] times it is a side effect of [illegible] drugs. About 10% of pregnant [illegible] 25% of people with iron deficiency experience this condition. A [illegible] dition may be associated with [illegible] caffeine. Patients should stop [illegible] take. Clonazine or diazepam [illegible] beneficial. SYN: *Ekbom's syndrome.*

restoration (rĕs″tō-rā′shŭn) [L. *restauro,* to fix] **1.** The return of [illegible] previous state. **2.** In dentistry, the [illegible] ment, material, or device that [illegible] tooth surface, or replaces a [illegible] the teeth and adjacent [illegible]

temporary r. A temporary [illegible] tooth cavity made from gutta [illegible] genol or some plastic material.

restorative (rĭ-stor′ă-tĭv) [L. *restauro,* to fix] **1.** Pert. to restoration. **2.** Anything that is effective in the regaining of health and strength.

restraint (rĭ-strānt′) [O. Fr. *restreindre*] **1.** The process of refraining from action, mental or physical. **2.** The condition of being hindered. **3.** In medicine, the use of major tranquilizers or physical means to prevent patients from harming themselves or others. In nursing, restraints are used in 36% to 86% of patients; in acute care hospitals, between 7.4% and 17% of patients are restrained.

The Food and Drug Administration, which regulates medical devices, has defined restraint as "a device, such as a wristlet, anklet, or other type of device, intended for medical purposes and used to limit its a patient's movements to the extent necessary for treatment, examination, or protection of the patient." Protective devices include safety vests, hand mitts, and wheelchair belts, body holders, [illegible] and protection nets.

[illegible] should be fitted properly [illegible] (neither too loose nor too tight). They [illegible] be applied in a manner that will [illegible] the patient from accidental self-[illegible] such as strangling or smothering [illegible] by slipping down in a bed, [illegible] or chair.

[illegible] are legally and ethically responsible for the safety and well-being of [illegible] in their care; however, when patient [illegible] or achieving the therapeutic [illegible] appears to require physical or [illegible] restraint, health care [illegible] must consider that such action [illegible] the patient's legal rights to autonomy [illegible] self-determination. Decisions to [illegible] physical or pharmacological restraint [illegible] must be based on a clear, identified [illegible] documented need for their use (i.e., [illegible] protecting the patient from harm or [illegible] part of the therapeutic goals cannot [illegible] met in any other manner.

[illegible] many patients, effective alternatives [illegible] physical restraint include providing [illegible] companionship and close supervision [illegible] explaining procedures to reduce [illegible] anxiety, when possible, removing [illegible] tubes, drains, and catheters to [illegible] discomfort and the potential for [illegible] entanglement; providing good lighting, ensuring [illegible] that pathways are clear, and that [illegible] is adequately secured to minimize [illegible] potential environmental hazards; [illegible] that the call button is easily accessible to facilitate patient requests for [illegible] assistance with ambulation; reducing unnecessary [illegible] distractions (e.g., background [illegible] noise) and enabling patient access to diversions [illegible] such as music and video movies [illegible] to encourage relaxation; and encouraging [illegible] ambulation and exercise to meet patient [illegible] needs for mobility.

[illegible] Informed consent must be obtained [illegible] from patient or guardian prior to use of [illegible] restraints. Restraints should not be used [illegible] without specific order from the treating [illegible] physician. Almost any type of restraint has [illegible] potential for harming the patient, thus [illegible] extremely important to monitor use and [illegible] ensure that it is applied correctly and removed [illegible] periodically.

[illegible] NURSING IMPLICATIONS: The nurse recognizes and reports patient behaviors that [illegible] indicate a need for restraint to ensure [illegible] safety and achievement of therapeutic [illegible] goals; describes nursing actions designed [illegible] to achieve care objectives without [illegible] resort to restraint and their effects; suggests [illegible] the minimum amount of restraint [illegible] needed to achieve the objectives of care [illegible] (e.g., restriction of mobility only to the degree [illegible] necessary); secures or reviews physician [illegible] orders for specific types of restraints; [illegible] validates informed consent; explains the use of the specific type of restraint to the patient and family member as a "reminder" needed for protection; and encourages verbalization of feelings and concerns and provides emotional support.

The nurse follows these general guidelines for application of restraints: the device that is most appropriate for the purpose is selected (e.g., padded mitts protect against patient removal of intravenous or other invasive tubing by limiting the ability to manipulate equipment with fingers but do not elicit the restlessness and frustration that occurs when the hands are tied down with wrist restraints). The status of tissues is assessed and documented before application. Bony prominences that will be in contact with the restraining devices are padded before application of such restraints. Restraints are applied to maintain a comfortable normal anatomic position, and mobility is limited only as much as is necessary to protect the patient (i.e., the nurse must change the position without defeating the objectives of the restraint). The nurse anchors restraint devices securely and ensures that they do not interfere with blood flow to the limbs or trunk; ensures that the restraints can be released quickly in the event of emergency; documents application and evaluation of current status; assesses and records the effects of the restraint on patient behavior, and on neurovascular status distal to the site; releases the restraint at frequent intervals (every 30 min); reports signs of increased agitation promptly; releases restraints (one at a time if the patient is unreliable or combative) and allows or provides range-of-motion exercises two to four times each shift; and evaluates the need for continuing restraint at least once per shift, discontinuing the devices as soon as the patient's status permits.

r. in bed The therapeutic use of physical means to prevent limb or body movement in bed. If a proper bed is not available, the following may provide a makeshift alternative. With the bed against the wall, straight-backed chairs are placed across the open side of the bed. They are tied in place by interlacing them with rope and then tying the rope to the foot and head of the bed. Another method is to place a wide board the length of the bed on the open side and to fasten it through three or four holes bored near the ends of the board. A sheet is folded lengthwise to the desired width, placed under the patient's body, and crossed in front below the arms. The hem ends are secured at the head and the side bar or the bedsprings. This allows some freedom for turning from side to side. The patient's hands and feet may be restrained by a clove hitch of wide bandage around the wrists and ankles and fastened to the side or foot of the bed.

NURSING IMPLICATIONS: The

+ *rhoia*, flow] **1.** Increased secretion of fat from the sebaceous glands of the skin. SYN: *seborrhea*. **2.** Fatty stools, as seen in pancreatic diseases.

idiopathic s. Sprue.

s. simplex Excessive secretion of the sebaceous glands of the face.

steatosis (stē″ă-tō′sĭs) [″ + *osis*, condition] **1.** Fatty degeneration. **2.** Disease of the sebaceous glands.

stegnosis (stĕg-nō′sĭs) [Gr. *stegnosis*, obstruction] **1.** Checking of a secretion or discharge. **2.** Stenosis. **3.** Constipation.

stegnotic (stĕg-nŏt′ĭk) Bringing about stegnosis. SYN: *astringent*.

Stegomyia (stĕg″ō-mī′ē-ă) A subgenus of mosquito of the genus *Aedes*, family Culicidae, suspected of transmitting the virus of yellow fever.

Steinert's disease (stīn′ĕrts) [Hans Steinert, Ger. physician, b. 1875] A hereditary disease characterized by muscular wasting, myotonia, and cataract. SYN: *myotonia dystrophica*.

Stein-Leventhal syndrome (stīn-lĕv′ĕnthăl) [Irving F. Stein, Sr., U.S. gynecologist, b. 1887; Michael L. Leventhal, U.S. obstetrician and gynecologist, 1901–1971] Hyperandrogenism with chronic anovulation in women without specific underlying adrenal or pituitary gland disease. Menses may be regular but later oligomenorrhea develops and then amenorrhea, but infrequently ovulation will occur. Infertility is usually persistent but may be treated with clomiphene, gonadotropins, or wedge resection of the ovary. SYN: *polycystic ovary syndrome*.

Steinmann's extension (stīn′mănz) [Fritz Steinmann, Swiss surgeon, 1872–1932] Traction applied to a limb by applying weight to a pin placed through the bone at right angles to the direction of pull of the traction force.

Steinmann pin A metal rod used for internal fixation of the adjacent sections of a fractured bone.

stella [L.] Star.

s. lentis hyaloidea Posterior pole of the crystalline lens of the eye.

s. lentis iridica Anterior pole of the crystalline lens of the eye.

stellate [L. *stellatus*] Star-shaped; arranged with parts radiating from a center.

stellate bandage A bandage that is wrapped on the back, crossways.

stellate cell Any cell that appears star-shaped (e.g., astrocytes and Kupffer's cells).

stellate fracture A fracture with numerous fissures radiating from the central point of injury.

stellate ganglion A sympathetic ganglion formed by the fusion of the inferior cervical and first thoracic ganglions.

stellate ligament One of the anterior costovertebral ligaments.

stellate reticulum The central cellular portion of the developing enamel organ, a nutritive store or protective covering of the developing enamel crown. SYN: *enamel pulp*.

stellate veins Stars of Verheyen.

stellectomy (stĕl-lĕk′tō-mē) [″ + *ektomē*, excision] The surgical removal of the stellate ganglion.

Stellwag's sign (stĕl′văgs) [Carl Stellwag von Carion, Austrian oculist, 1823–1904] Widening of the palpebral aperture with absence or lessened frequency of winking, seen in Graves' disease.

stem 1. [AS. *stemn*, tree trunk] Any stem-like structure. **2.** To derive from or originate in. **3.** [ME. *stemmen*] To check, stop, or hold back.

stem cell Hemocytoblast.

stenion (stĕn′ē-ŏn) [Gr. *stenos*, narrow] The craniometric point at the extremities of the smallest transverse diameter in the temporal region.

steno- [Gr. *stenos*, narrow] Combining form meaning *narrow* or *short*.

stenobregmatic (stĕn″ō-brĕg-măt′ĭk) [″ + *bregma*, front of head] A term applied to a skull with narrowing of the upper and frontal portions.

stenocephaly (stĕn″ō-sĕf′ă-lē) [″ + *kephalē*, head] Narrowness of the cranium in two or more diameters.

stenocompressor (stĕn″ō-kŏm-prĕs′ŏr) [″ + L. *compressor*, that which presses together] An instrument for compressing Stensen's ducts to stop the flow of saliva.

stenopaic, stenopeic (stĕn-ō-pā′ĭk) [Gr. *stenos*, narrow, + *ope*, opening] Provided with a narrow opening or slit; said of devices to protect against snow blindness.

stenosal (stē-nō′săl) [Gr. *stenos*, narrow] Stenotic.

stenosed (stē-nōst′, stĕn′ōzd) Marked by stenosis; constricted.

stenosis (stē-nō′sĭs) [Gr., act of narrowing] The constriction or narrowing of a passage or orifice. SYN: *stricture*.

ETIOLOGY: This may result from embryonic maldevelopment, hypertrophy and thickening of a sphincter muscle, inflammatory disorders, or excessive development of fibrous tissue. It may involve almost any tube or duct.

aortic s. SEE: under *aortic*.

cardiac s. A narrowing or constriction of any of the orifices leading into or from the heart or between the chambers of the heart.

cicatricial s. Stenosis resulting from any contracted cicatrix.

lumbar s. An overgrowth of the laminae of the vertebrae so that the spinal canal is narrowed. This may cause back and leg pain, esp. when walking. SEE: *back pain*.

mitral s. Narrowing of the mitral orifice, obstructing free flow from the atrium to the ventricle. SEE: *Nursing Diagnosis Appendix*.

pulmonary s. Narrowing of the opening